

THERMOTOLERANT CAMPYLOBACTER AND CAMPYLOBACTER INDUCED ENTERITIS

By
MATS WALDER



From the Department of Medical Microbiology, University of Lund,
Malmö General Hospital, Malmö, Sweden
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ENTERITIS

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Mats Walder
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The phenotypic properties and genetic relationship between strains of thermotolerant Campylobacter (CJC) was studied. A serological method to detect antibodies to CJC without access to the infecting strain was developed. The in vitro susceptibility of CJC strains to antibiotics was studied and the clinical course and epidemiology of CJC enteritis in a Swedish population were investigated. Human isolates were in 87 % C.jejuni (hippurate positive), and in 13 % C.coli (hippurate negative). C.jejuni and C.coli formed separate, homogenous DNA homology groups except nalidixic acid resistant strains of C.coli (NARTIC) which were genetically different from C.coli. Porcine isolates were solely C.coli as were 93 % of canine isolates. Of dog strains 2/3 had negative or weak catalase reaction (CNW) and were genetically different from all other strains. Analyzing immune response in enteritis patients with campylobacter whole cell IgG and IgM ELISA 94 % were positive compared to only 5 % of healthy controls. Children had detectable levels of antibodies suggesting early immunization. In vitro CJC strains were most susceptible to doxycycline, erythromycin and gentamicin. All strains were resistant to β-lactam antibiotics. In 13 % of enteritis patients antibiotic treatment was given. After therapy CJC could not be recovered in stools. The incidence for CJC in 8991 enteritis patients was 8,3 %. CJC was the most common proven bacterial etiology in acute enteritis. About half of the enteritis patients (53%) had acquired their infection in Sweden. CJC enteritis was not a severe disease and only 11 % were admitted to hospital. Four patients (2%) with reactive nonseptic arthritis were seen. CJC was isolated from 6 % of Swedish enteritis children. Children were less severely affected than adults. The major source of Campylobacter infection was chicken especially by cross-contamination from an uncooked to a cooked dish. Strains from dogs and pigs were often different compared to human strains. Person to person spread was not observed.		
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From the Department of Medical Microbiology, University
of Lund, Malmö General Hospital, Malmö, Sweden

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This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I Sandstedt, K., Ursing, J. & Walder, M.
Thermotolerant Campylobacter with no or weak catalase activity isolated from dogs. Submitted for publication.
- II Walder, M., Sandstedt, K. & Ursing, J.
Phenotypical characteristics of thermotolerant Campylobacter from human and animal sources. Submitted for publication.
- III Ursing, J., Walder, M. & Sandstedt, K.
Base composition and sequence homology of deoxyribonucleic acid of thermotolerant Campylobacter from human and animal sources. Submitted for publication.
- IV Walder, M.
Susceptibility of Campylobacter fetus subsp jejuni to twenty antimicrobial agents. Antimicrob Agents Chemother 16:37-39, 1979.
- V Walder, M. & Forsgren, A.
Enzyme-linked immunosorbent assay (ELISA) for antibodies against Campylobacter jejuni and its clinical application. Acta Pathol Microbiol Scand (B) In press.
- VI Walder, M.
Epidemiology of campylobacter enteritis. Scand J Infect Dis 14:27-33, 1982.
- VII Walder, M., Lindberg, A., Schalén, C. & Öhman, L.
Five cases of Campylobacter jejuni/coli bacteremia. Scand J Infect Dis 1982. In press.
- VIII Persson, B.L., Thorén, B., Tufvesson, B. & Walder, M.
Diarrhoea in Swedish infants. Acta Paediatr Scand 71, 1982. In press.
- IX Thorén, A., Stintzing, G., Tufvesson, B., Walder, M. & Habte, D.
Aetiology and clinical features of severe infantile diarrhoea in Addis Ababa, Ethiopia. J Trop Pediatr 28:127-131, 1982.

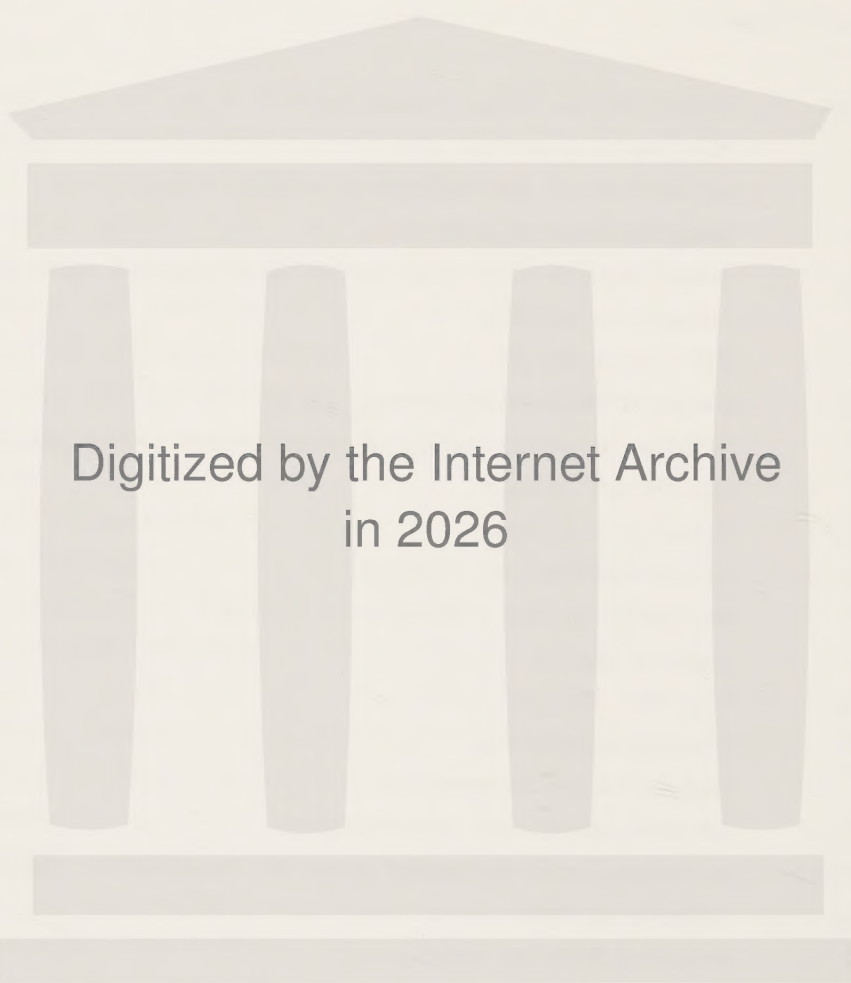
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ABBREVIATIONS

Cf ss	Campylobacter fetus subspecies
CFT	complement fixation test
CIP	collection of the Pasteur Institute, Paris
CJC	Campylobacter jejuni/coli
CNW	campylobacter with negative or weak catalase reaction
CRP	c-reactive protein
DIG	diffusion-in-gel
ELISA	enzyme-linked immunosorbent assay
FBP	ferrous sulphate, sodium bisulphite and sodium pyruvate
G+C	guanine and cytosine
H-ag	flagellar-antigen
HLA	histocompatibility antigen
HUS	hemolytic-uremic syndrome
Ig	immunoglobulin
K-ag	capsular antigen
LPS	lipopolysaccharide
LT	thermo-labile enterotoxin
MIC	minimum inhibitory concentration
NARTC	nalidixic acid resistant group of thermophilic campylobacter
O-ag	somatic antigen
RBR	relative binding rate
SOD	superoxide dismutase
ST	thermo stable enterotoxin
Tm(e)	thermal elution midpoint
TTC	2,3,5-triphenyltetrazolium chloride
V	vibrio



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INTRODUCTION

Campylobacter species cause mainly zoonotic diseases known to veterinarians since the early 20th century. Two species cause human disease, *C. fetus* ss *intestinalis* and *C. jejuni*. The latter is the causative agent of diarrhoea affecting previously healthy persons. It was undetected for many years since the techniques traditionally used in clinical laboratories did not permit the growth of these fastidious bacteria.

As campylobacter enteritis is a "new" human disease, several questions have to be answered:

How to isolate, identify and characterize the microorganism? What are the sources of origin and how is the genetic relationship between human and animal strains? What are the clinical manifestations of campylobacter infection, and are there any characteristic symptoms and signs in campylobacter enteritis? How common is it? Which individuals are affected? What are the sources and routes of infection? Is there immunity in the general population and after disease in patients?

In order to find an answer to these questions I studied the bacteriological, immunological, clinical and epidemiological course of the human cases in Malmö, Sweden, between December 1977 and December 1981.

HISTORY

Infection with *Vibrio fetus* was primarily the concern of medical veterinarians and it is therefore necessary to begin with a very brief résumé of the bacteriological work done by them.

A vibroid-shaped organism was first described in 1909 by McFaydean and Stockman as a cause of infectious abortion in cattle and sheep. These bacteria, later named *Vibrio fetus*, caused economic loss to farmers because they impaired the fertility of the cattle. The disease was considered venereal, carried by the bull in the testis for life, where it caused no signs of disease. *V. fetus* also caused abortion in sheep, but the mode of transmission was different. Contaminated food and water were suspected as transmitting agents.

V. fetus was not suspected to be pathogenic for man until 1947, when Vincent et al. published 2 cases of abortion in women caused by *V. fetus* that were isolated from blood. Since then, about 110 human cases of infections with *V. fetus* have been described, the most common form being a pure septicemia without dissemination or splenomegaly but with fever as a constant feature (Butzler 1978). This vibrio now called *Campylobacter fetus* ss *fetus* (Sebal and Veron 1963) is recognized as an opportunist that mainly attacks debilitated patients with impaired defence against infections (Bokkenheuser 1970).

Enteritis, another kind of human disease associated with "bovine vibrios" was first described by Levy in 1946. This disease is characterized by nausea, abdominal cramp, diarrhoea, fever, general malaise and headache. Levy reported of a putative milk-borne outbreak of enteritis affecting 357 inmates at two prisons in Illinois, USA. Vibrios that fit the description of *Campylobacter* were seen in 31 of 73 mucoid stools and in 13 of 39 blood cultures from patients but were cultured from the blood of only 4 of the patients.

A similar report of 4 cases of gastroenteritis caused by a thermotolerant closely related vibrio species designated "related vibrios" was presented by King in 1957. The microorganisms were isolated from the blood of four children of whom three had severe diarrhoea and two had frank blood in the stools.

Except for the odd chance isolation, "related vibrios" as a cause of enteritis were for many years undetected because the techniques traditionally used in clinical laboratories did not fulfill the growth requirements for these fastidious bacteria. The few successful isolations were all obtained from blood or other normally sterile sites free from competing organisms (Middelkamp and Wolf 1961, Wheeler and Borchers 1961, Mandel 1963, Darell et al. 1967). In these reports the suspicion was put forward that the organisms were present in the gut and that the infection was not as rare as the few reports suggested. Attempts to prove this were made impossible by the lack of suitable selective culture techniques. Since vibrios were well known in veterinary practice as a cause of infection in cattle and sheep an adequate isolation technique was eventually developed.

Cooper and Slee in 1971 isolated *Vibrio fetus* from faeces of an enteritis patient using selective antibiotic discs. Dekeyser et al. (1972) in Brussels made the first isolation of the "related vibrios" from the stools of two enteritis patients by using a 0.65 μ m Millipore filter technique developed by Plumer et al. 1962. The Brussel team (Butzler et al. 1973) showed that "related vibrios" could be isolated from 5% of children and from 4% of adults with diarrhoea. However, it was not until the presentation of a selective

culture medium for faeces by Skirrow in 1977 that the bacteria, at that time called *Campylobacter jejuni*, were easily cultured and later recognized world wide as a cause of human enteritis.

AIMS OF THE STUDY

The purpose of the present investigation was:

- to characterize the phenotypic properties of strains of thermotolerant campylobacter (CJC) from different sources of origin
- to determine the genetic relationship between strains with different phenotypic characteristics
- to develop a method for detection of antibodies to CJC and analyze the immuneresponse in sera from patients with campylobacter enteritis and to estimate the immunity in the population
- to study the in vitro susceptibility of campylobacter strains to antimicrobial drugs
- to study the clinical course and epidemiology of campylobacter enteritis in a Swedish population
- to investigate campylobacter enteritis in Swedish and Ethiopian infants

These points are discussed in the following chapters based on papers referred to by their Roman numerals I-IX, own unpublished results and results published by others.

BACTERIOLOGY

Taxonomic review up to 1974

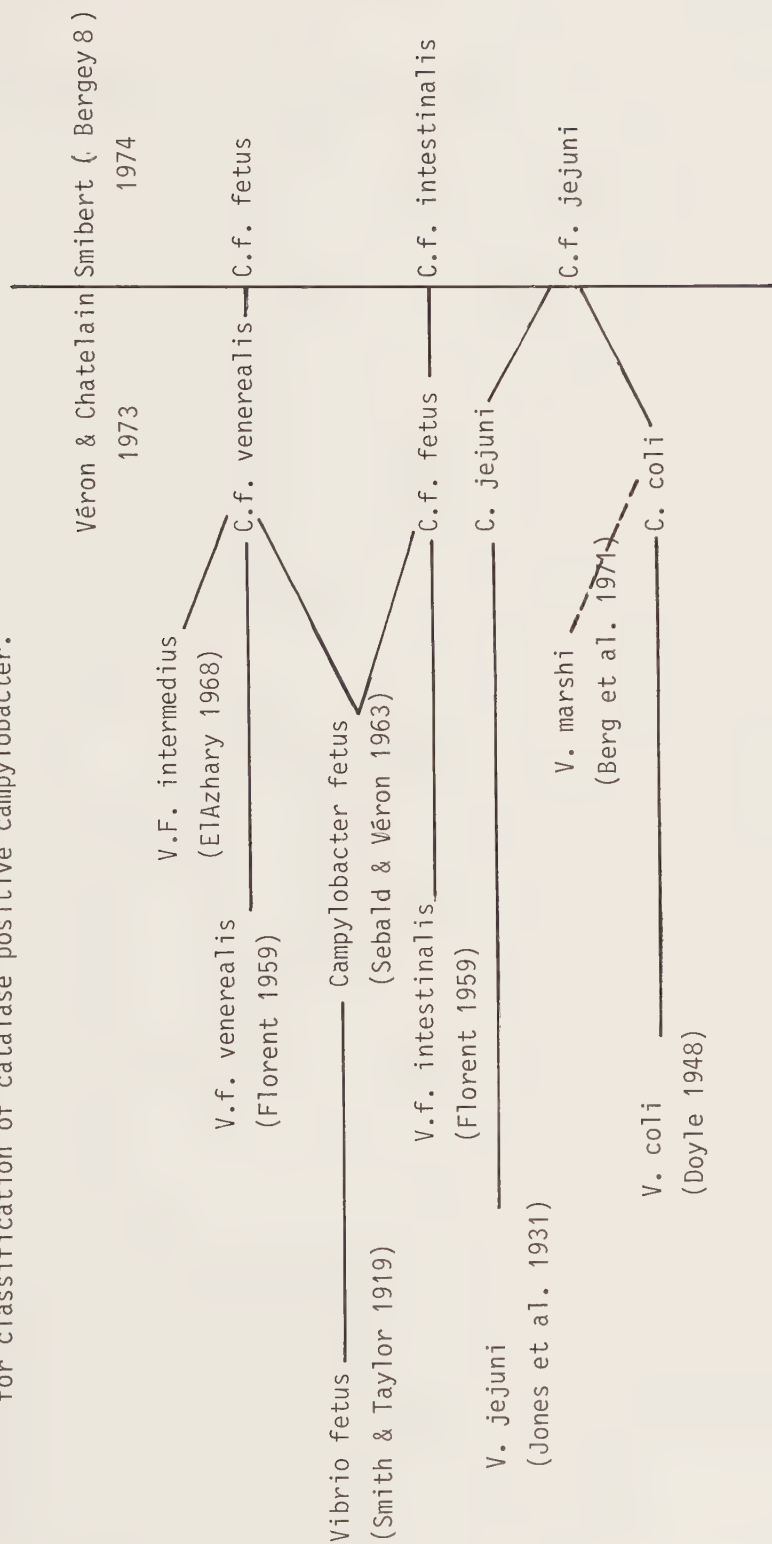
Smith and Taylor in 1919 isolated an oxidase positive curved motile gram negative rod associated with ovine and bovine abortion. It was originally placed in the genus *Vibrio* and named *Vibrio fetus*. (Fig. 1).

Florent in 1959 separated *Vibrio fetus* into two subspecies; *Vibrio fetus* ss *venerealis* being the agent of enzootic sterility and cause of abortion in cattle, and ss *intestinalis* responsible for sporadic cases of abortion in cattle and enzootic abortion in sheep.

Vibrio fetus does not grow at 42°C , but a thermotolerant species of vibrio was recognized by King already in 1957 and was called "related vibrio" or high temperature vibrio. These bacteria may be identical with those described by Jones et al. in 1931 as the causative agent of winter dysentery in cattle and named *Vibrio jejuni*. Another similar organism causing dysentery in swine was named *Vibrio coli* by Doyle in 1948. A disease in chicken designated as avian infectious hepatitis was in 1958 described by Peckham to be due to a vibrio similar to the "related vibrios" described by King.

In 1963 Sebald and Véron proposed the new genus *Campylobacter*, a name derived from the Greek "campylo" (curved) and "bacter" (rod). The reason was that the "true" vibrios differ greatly compared with *campylobacter* in phenotypic respects e.g. in the ability to ferment selective sugars with acid production and in guanine and cytosine (G + C) content of the deoxyribonucleic acid (DNA) indicating no genetic relationship. The new genus was

Historical development of the taxonomic nomenclature for classification of catalase positive campylobacter.



Figur 1

divided into two groups according to the ability of the organism to produce catalase. Catalase negative campylobacter are not known to be pathogenic for man (Smibert 1978). The catalase positive group consists of *Campylobacter fetus* as the type species of the genus and *C. jejuni*, the closely related set of organisms first recognized by King and later called "related campylobacter".

In 1973 Véron and Chatelain made a taxonomic study of the genus campylobacter suggesting the type species *C. fetus* being divided into two subspecies; *C. fetus* ss *fetus*, and *C. fetus* ss *venerealis*. The former is responsible for infectious abortion in cattle and sheep and also causes disseminated infection in human compromised hosts. *C. fetus* ss *venerealis* is responsible for enzootic venereal sterility in cattle. Two other species were described; *Campylobacter coli* and *Campylobacter jejuni*, both of the related high temperature group of campylobacter found predominantly in pigs and chickens respectively, now known to cause human enteritis. Smibert in Bergey's manual (1974) brought the two species together to *C. fetus* ss *jejuni*, because the original *C. coli* strain was poorly characterized and representative strains were not available for study.

Phenotypic classification

As previously mentioned Véron and Chatelain regarded the "related" group of campylobacter as consisting of two species and the difference between *C. jejuni* and *C. coli* was based mainly on different degrees of tolerance to TTC and to 8% glucose (Véron and Chatelain 1973).

Skirrow and Benjamin (1980a) made a rough grouping of campylobacter strains from different animal and human sources. The jejuni/coli groupings were based mainly on tolerance to TTC and growth at 30.5°C and 45.5°C finely balanced tests that were unsuitable for routine use. The CJC strains could be divided into two groups with results resembling the type strains *C.jejuni* and *C. coli*, but some strains had intermediate characters. Moreover, some CJC strains were nalidixic acid resistant (no growth inhibition with 30 µg disc). These strains formed a distinct CJC subgroup derived from local seagulls and Skirrow designated them nalidixic acid resistant group of thermophilic campylobacter (NARTC) (Table 1).

Table 1. Distribution of CJC biotypes in per cent among different animals according to Skirrow (1980a)*. (Hippurate test was not used)

	Cow	Sheep	Pig	Hen	Dog+cat	Seagull	Man
<i>C. jejuni</i>	95	80	5	75	88	55	95
<i>C. coli</i>	5	20	94	23	4	-	5
NARTC	-	-	1	2	8	45	-

*modified by Skirrow, M.B., personal communication

Harvey in 1980 was able to distinguish *C. jejuni* from *C. coli* by a simple test of hippurate hydrolysis later applied by Skirrow and Benjamin (1980c) (Table 2). They also reported that resistance to nalidixic acid was not confined to *C. fetus ss fetus* and *ss venerealis* but some CJC strains that were negative in hippurate hydrolysis were also nalidixic acid resistant (NARTC). In the same study Skirrow and Benjamin detected another subgroup *C. jejuni* II that produced H₂S when growing in iron medium (Table 2).

Table 2. Tests for discriminating among catalase positive campylobacter according to Skirrow (1980c)

	<u>C.coli</u>	<u>C. jejuni I</u>	<u>C. jejuni II</u>	<u>NARTC</u>	<u>C. fetus</u>
Growth at 42°C	+	+	+	+	-
Growth at 25°C	-	-	-	-	+
Sensitivity to nalidixic acid	+	+	+	-	-
Hippurate hydrolysis	-	+	+	-	-
H ₂ S in iron medium	-	-	+	+	-

Leaper and Owen in 1981 published a study of assay results on chemical tests and cellular fatty acid content in campylobacter. The hippurate hydrolysis test was the only reliable means to differentiate between *C.coli* and *C. jejuni*. They concluded that it is justifiable to recognize *C. fetus* and *C. jejuni* as separate species but the taxonomic status of *C. coli* needed further attention and they considered it was realistic to view *C. coli* as a subspecies of *C. jejuni*. Luechtefeld and Wang (1982) using both TTC and hippurate hydrolysis test reported that ability to hydrolyze hippurate was seen in 99% of human isolates, 76% of avian isolates, 100% of cattle and dog isolates and none of pig isolates. Resistance to TTC was detected in 97% of the human isolates, 95% of the avian isolates and 100% of the dog, cattle and pig isolates. In no case did the isolates show both lack of ability to hydrolyze hippurate and sensitivity to TTC.

Since we have not separated *C. jejuni* and *C. coli* consistently through the studies since 1977, I have designated the thermotolerant group of "related" campylobacter as *Campylobacter jejuni/coli* (CJC).

At the first international Workshop on *Campylobacter* infections in Reading 1981 it was decided to adopt the nomenclature of Véron and Chatelain(1973).

In our study (II) of phenotypic characteristics of thermotolerant catalase positive campylobacter good reproducibility for the hippurate hydrolysis test and nalidixic acid resistance was obtained. Using these tests supplemented with discriminating tests for tolerance to 2,3,5-triphenyl-tetrazolium chloride (TTC) and resistance to metronidazole we examined 168 human and 146 animal isolates. All strains grew readily at 37°C except the 17 broiler strains which initially only grew at 42°C. This might suggest an adaption to warm-blooded animals such as poultry. Following subcultivation these strains grew well at both 37°C and 42°C.

Most isolates were found to be *C. jejuni*; hippurate positive, sensitive to nalidixic acid and resistant to TTC. Human strains were in 87% *C. jejuni* i.e. hippurate positive. Three *C. jejuni* strains were resistant to nalidixic acid and another three were sensitive to TTC. Among human *C. coli* (hippurate negative) strains we found no NARTC (nalidixic acid, TTC and metronidazole resistant) strains but two were TTC sensitive. Thus totally 97% of human CJC strains were TTC resistant.

All porcine isolates were hippurate negative *C. coli* and 5 strains were nalidixic acid resistant, NARTC-like, but they were metronidazole sensitive.

In cattle 2/6 strains were *C. coli* (hippurate negative) and nalidixic acid resistant but they were sensitive to both TTC and metronidazole. All isolates from sheep abortion were *C. jejuni*. Among poultry strains 2/3 were *C. jejuni* and so were all broiler isolates. Together poultry, broiler and wild bird isolates were distributed on both species. However, one avian isolate from a diver was a true NARTC strain.

In study I we found that 63 out of 98 canine isolates had catalase negative or weak reactions (CNW). The CNW strains were all hippurate negative and nalidixic acid sensitive. This phenotypic pattern was not seen in any other CJC strain.

Our results demonstrated that *C. jejuni* was the species most commonly isolated from humans and it was the only species recovered from broilers and from sheep abortion. *C. coli* was the dominating species isolated from pigs. The avian strains were distributed on both species. Of the isolates recovered from dogs a majority represented a phenotype not previously described.

Genetic studies of the genus Campylobacter

Some uncertainty exists about the genetic relationships between and within the various campylobacter species because they are biochemically relatively inactive.

Comparative studies (I) (III) of DNA base composition (mol% G + C) and base sequence similarities between type and reference strains of various species of campylobacter from human and animal sources were performed.

A short description of DNA base composition estimation. When DNA in aqueous solution is heated, the complementary strands separate so that the double-stranded helix (native configuration) changes to single stranded DNA (denaturated configuration). This is accompanied by an increase in the absorbance at 260 nm of the nucleotide base (hyperchromic effect). The thermal denaturation midpoint (T_m) was calculated graphically after monitoring the increase in absorbance as a function of temperature. The base composition was computed according to formula (I) assuming the mol% G + C of E. coli B to be 52.0.

We studied the DNA base composition (I, III) of the various campylobacter species type strains (CIP) and found the following mean mol% G + C contents (Table 3), *C. fetus* ss fetus 34.3, *C. coli* 32.4, *C. jejuni* 31.6 and *C. spurtorum* ss bubulus 31.0. Our results of G + C contents agree well with previously reported values on the same strains (Owen and Leaper 1981, Véron and Chatelain 1973).

Table 3. DNA base composition (mol% G + C) thermotolerant *Campylobacter* and for type and reference strains

	Number	Mean mol% G + C	Range
<i>C. jejuni</i>	12	31.9	31.2 - 32.4
CIP 702 (type strain)	1	31.6	
<i>C. coli</i>	10	32.6	32.1 - 33.1
CIP 7080 (type strain)	1	32.4	
<i>C. sp.</i> (NARTC)			
NCTC 11352 (ref. strain)	1	31.4	
<i>C. sp.</i> (NARTC-like)			
C 120 C 269	2	35.3	
<i>C. sp.</i> (CNW) C 303			
192, 219, 231, 283	5	35.5	35.2 - 35.8
<i>C. fetus</i> ss . fetus			
CIP 5396 (type strain)	1	34.3	
<i>C. sputorum</i> ss . bubulus			
CIP 53103 (type strain)	1	31.0	

sp = species,

The mean G + C contents of *C. jejuni* and *C. coli* (III) from different sources of origin were very similar (Table 3). Mean mol% G + C for *C. coli* (32.6) was separated from the nalidixic acid resistant *C. coli* NARTC reference strain (31.4) and even more different to Swedish NARTC-like strains (35.4). However, *C. jejuni* (mean% G + C 31.9) was similar to nalidixic acid resistant *C. jejuni* (32.2). Mol% G + C was also determined (I) for 5/63 CNW strains (mean 35.5) and 2/28 catalase positive hippurate negative

strains (32.7). The CNW dog strains formed a genetically separated group with G + C contents more than 3% higher than for any other thermotolerant strain.

A short description of the DNA-DNA hybridization technique. Determination of the extent that single-strand DNA molecules from two different bacterial strains will pair to form double-strand interstrain duplexes in vitro is a widely used taxonomic tool. The technique of DNA-DNA hybridization or re-association enables estimates to be made of the relative number of nucleotide sequences held in common by two microorganisms. This in turn is generally assumed to be a measure of their overall genetic relatedness. Low values for the differences in thermal elution midpoint ($\Delta T_m(e)$) also indicate high homogeneity.

Basden et al. 1968 studied the relatedness among six bacterial strains classified as vibrios but with phenotypic characteristics of *Campylobacter*. They found heterogeneity amongst these unnamed *Campylobacter*-like organisms. Owen and Leaper (1981) studied DNA characteristics of type and reference strains. The *C. jejuni* and *C. coli* type strains were unrelated and they can be considered as belonging to separate species. They conclude that the species of the genus *Campylobacter* as described by Véron and Chatelain (1973) are not contradicted by their DNA results.

In our DNA-DNA hybridization study (III) the range of homologous binding was 75-85%. The relative binding rate (RBR) at 60°C was calculated as percentage of homologous binding after correction for self binding of labelled DNA.

The results of study III (Table 4) indicate that *C. jejuni* and *C. coli* (when the hippurate test is used as differentiating criterium) irrespective of source of origin make up genetically homogenous and separable bacterial groups. Moreover, they seem to be more genetically related to each other than to any of the campylobacter species studied.

Nalidixan resistant *C. jejuni* are genetically similar to sensitive *C. jejuni*. However, concerning resistant *C. coli* i.e. the nalidixic acid resistant group of thermotolerant campylobacter (NARTC) this group was genetically heterogenous. The NARTC reference strain showed a higher degree of relatedness to labelled *C. jejuni* and *C. coli* than Swedish NARTC-like strains. In study I the 5 dog strains CNW were found to be homogenous and the strain labelled had a DNA relatedness to *C. jejuni* and *C. coli* of about 40%. The binding rates against *C.f. ss fetus* and *C.s. ss bubulus* were considerably lower than against the thermotolerant species but they nevertheless indicate some degree of genetic relationship. Although, the CNW isolates seem to make up a good genospecies, we feel that the phenotypical differentiation should rely on more than one property.

However, in the future there will most likely be described new strains that deserve status as a species.

Identification

An attempt to separate campylobacter by their appearance in microscope was made by Karmali et al. (1981a). They examined catalase-positive campylobacters by phase contrast microscopy and found significant differences in cell size, amplitude and wavelength between the spiral forms of the three groups, *C.f. ss fetus*, *C.f. ss venerealis* and CJC.

Table 4. DNA relatedness for thermotolerant Campylobacter and for type and reference strains at 60°C (mean values)

	C.jejuni M 18		C.jejuni CIP 702		C.coli M 5		C.coli CIP 7080	
	RBR (r)	$\Delta T_m(e)(r)$	RBR (r)	$\Delta T_m(e)(r)$	RBR (r)	$\Delta T_m(e)(r)$	RBR (r)	$\Delta T_m(e)(r)$
C.jejuni number	10	10	9	8	8	8	3	3
	94	2.1	91	2.0	58	8.9	57	9.0
	(86-100)	(1.4-2.8)	(81-100)	(1.8-2.3)	(47-69)	(7.1-10.1)	(52-61)	(8.6-9.2)
C.coli number	4	4	2	2	10	10	2	2
	64	8.5	62	8.2	89	1.0	86	1.4
	(59-67)	(8.1-8.8)	(55-68)	(8.5-8.9)	(81-97)	(0.3-1.9)	(85-86)	(1.4)
C.sp.NARTC								
NCTC11352	40	11.9	29	-	-	-	26	-
(ref str)								
C.sp.NARTC-like	1	-	2	-	2	-	1	-
C 120 C 269	17	-	16(15-17)	-	10(<10-11)	-	(<10)	-
C.sp.CNW	2	2	2	2	2	2	2	-
C.231 C 303	41	15.2	42	15.4	44	12.8	27	-
	(36-46)	(15.0-15.4)	(40-43)	(15.3-15.4)	(44)	(11.6-13.9)	(26-27)	-
C.fetus ss fetus								
CIP5396	24	-	24	-	-	-	28	-
(type str)								
C.sputorum ss								
bubulus CIP53103	25		24	-	-	-	25	-
(type str)								

r = range; sp = species

At our laboratory all colonies suspected to be CJC by sight on the selective agar plates (Skirrow's supplement) were tested for oxidase and catalase activity. If positive, a gram stain was performed to identify the characteristic gulwing or comma shape.

In microscope campylobacters are gram negative, curved spiral rods about 2 μm long by 0.3 μm width and the ends of the cell are usually pointed (Unpublished results). After 4-6 days in cultures the cells may become coccoid and coccoid cells appear to be a degenerative form because cultures composed mainly of coccoid forms are non viable.

After microscopy a preliminary diagnose of CJC could be made. To confirm the diagnosis the susceptibility to nalidixic acid was tested. Sensitivity to nalidixic acid is a simple verification test for CJC, but there are a few resistant strains both in the *C. jejuni* group and among *C. coli*, the latter designated NARTC (nalidixic acid resistant group of thermophilic campylobacter) (Skirrow and Benjamin 1980c). Furthermore, a test of motility was made either in a microscope with hanging drop or in a motility agar broth with 0.2% agar. It has been shown that growth or absence of growth at 25 $^{\circ}\text{C}$ is a more reliable characteristic in differential diagnosis of CJC than growth at 42 $^{\circ}\text{C}$ as some strains of *C. fetus* are capable of growing in the latter temperature (Smibert and Von Graevenitz 1980, Skirrow and Benjamin 1980b). Therefore all our strains were tested for growth at 25 $^{\circ}\text{C}$ microaerophilically and for growth aerobically.

All our CJC strains grew at 42°C and 37°C and none grew at 25°C (II).

Among our 837 CJC strains isolated during 4 years we did not find any belonging to NARTC group, but 2% of *C. jejuni* were resistant to nalidixic acid.

Growth requirements for CJC

CJC does not metabolise carbohydrates and it can neither phosphorylate nor transport glucose. Its energy comes from the tricarboxylic acid cycle. A few amino acids such as oxalacetic acid and aspartic acid can be deaminated (Smibert 1978).

CJC requires a low concentration of oxygen for growth, yet it is inhibited by oxygen at normal atmospheric partial pressure (21%) and some strains in candle jar (17% O₂) (Smibert 1978). The optimum concentration of oxygen has been reported to be 5%. There is a requirement for carbon dioxide (Kiggins and Plastringe 1956).

High energy radicals such as superoxide and peroxide are produced in culture media when exposed to light and air. The aero-tolerance of campylobacter was reported to be increased by addition of ferrous sulphate, sodium bisulphite and sodium pyruvate (FBP) to the medium (George et al. 1978). Iron and bisulphite together act non enzymatically to destroy superoxide radicals whereas pyruvate can destroy hydrogenperoxide. Catalase and superoxide dismutase (SOD) have been shown to aid growth by reducing medium toxicity and are both produced in the cells of campylobacter (Smibert 1978).

We have observed that CJC grows in thioglycollate medium or any peptone yeast extract medium but it is no ample growth (Unpublished results). It grows

better in a semisolid medium (0.14-0.16% agar) when incubated aerobically. Growth starts a few mm below the surface as a band which becomes thicker until it is 4-5 mm thick. It grows only at the top of the medium.

The problem with large scale cultures is to attain adequate cell yields. We have found that high inoculum of cultures shaken in bottles with the atmosphere adjusted to the optimum concentrations and with the addition of FBP, KNO_3 , formate, fumarate (Smibert and Holdeman 1976) and lactate to a medium will give the highest cell yields (Unpublished results). However, because of the risk of contaminating the broth we made most of our large scale cultures by subculturing to 200 blood agar plates which were incubated microaerophilically and then harvested with saline or broth.

Techniques for isolation

The isolation of campylobacters from faeces requires special selective techniques which depend either on differential filtration or direct inoculation on agar containing selective antibiotics. The filtration method used by Dekeyser et al. (1972) is too tedious for routine use. In brief, faecal material was diluted with nutrient broth. After homogenization and sedimentation the supernatant was centrifugated. The surface liquid (4 ml) was sucked into a syringe for subsequent filtration through a $0.65 \mu\text{m}$ Millipore filter. The first 3 ml was discarded, and 0.3 ml of the remaining liquid was filtered directly onto two agar dishes.

The selective agar method now widely used, deals with three compositions of antibiotic supplements to the agar. They are called Skirrow's medium (1977),

Butzler's medium (1979) and Blaser-Wang medium (Blaser et al. 1980c) and their compositions are shown in Table 5. All contain 7-15% horse or sheep blood.

In our studies (I-IX) human blood peptone agar plates supplemented with Skirrow's selective antibiotics have been used for isolation of strains from stool. Most subcultures were made on human blood peptone agar plates without antibiotics.

Table 5. Selective culture media

<u>Skirrow's medium</u>	<u>Blaser-Wang medium</u>	<u>Butzler's medium</u>
Vancomycin 10 mg/l	Vancomycin 10 mg/l	Bacitracin 25 IU/l
Polymyxin B 2 500 IU/l	Polymyxin B 2 500 IU/l	Novobiocin 5 mg/l
Trimethoprim 5 mg/l	Trimethoprim 5 mg/l	Colistin 10 000 IU/l
	Cephalothin 15 mg/l	Cefazolin 15 mg/l
	Amphotericin B 2 mg/l	Acti-dione 50 mg/l (cycloheximide)
5-7% lysed horse blood	10% sheep blood	15% sheep blood
Oxoid BA Base No.2	Brucella agar (BBL)	Thioglycollate agar

Campylobacter grow microaerophilically in oxygen concentrations round 5% (Kiggins and Plastringe 1956). In our laboratory, this atmosphere was obtained by drawing a partial vacuum of 500 mm Hg in an iron anaerobic jar without catalyst and then substituted with a gas mixture of 91% N₂, 4% H₂

and 5% CO₂. All strains were isolated at 42⁰C but subcultures were performed at 37⁰C, both cultures were incubated for 48 h. During these circumstances isolates of CJC appeared as grey, flat effuse drops on the agar sometimes with profuse swarming especially if the atmosphere was damp.

Survival in biological milieus

Luechtefeld et al. found in 1981 that CJC survived in feecal specimens for an average of 4 days at 25⁰C, 9 days at 4⁰C and for over 24 days in transport medium at 4⁰C. However, after freezing at -20⁰C for 24 h, 80% of feecal samples were negative and similar results were obtained with specimens frozen at -70⁰C. Of six transport media tested, 80% of the samples survived for over 18 days in Cary-Blair medium with decreased agar concentration (0.16%) held at 4⁰C. Survival in Cary-Blair medium was also reported to be superior to buffered glycerol saline and modified Stuart transport medium. The survival in fresh food and biological milieus has also been investigated by Blaser et al. (1980c), Grant et al. (1980) and Svedhem et al. (1981a). They all found that maximal viability of CJC was obtained if strains were held at 4⁰C. It made no difference if the bacteria were kept in intestinal contents, food, milk, water or urine. Heat treatment at 60⁰C for 15 minutes rapidly killed CJC strains.

In our laboratory we found that CJC survived in feecal specimens for about 7 days at room temperature and 4 days longer at 4⁰C. In Stuart transport medium kept at 4⁰C, CJC survived for more than 2 weeks. Awaiting lyophilization we kept our strains frozen at -18⁰C in buffered glycerol saline up to six months without loosing any strains (Unpublished results).

Antibiotic susceptibility

Vanhoof et al. 1978 reported 8% of CJC strains resistant to erythromycin and later altered this to 2.3% (Vanhoof et al. 1980). From Canada (Karmali et al. 1981b) and England (Brunton et al. 1978) 0.5-1% of strains have been reported to be erythromycin resistant.

Analyzing the sensitivity to tetracycline Vanhoof et al. (1978) found 5% and Karmali et al. (1981b) 10% of the strains to be resistant to this drug. Resistance to tetracyclines has been shown to be transmitted by plasmids (Taylor et al. 1981, Austen and Trust 1980).

CJC appears to be very sensitive to furazolidone, an oral nitrofurantoin derivative with minimal absorption (Butzler et al. 1974, Vanhoof et al. 1978) and there seems to be no resistance.

In 1979 we studied the susceptibility of 100 CJC strains to 20 antimicrobial agents by agar dilution test (IV) and found 8 strains resistant to erythromycin. In 1980 when 435 strains of CJC were assayed by disc diffusion test (VI) only 3 more strains were resistant to erythromycin, thus constituting a total resistance of 2.6% of the strains (Table 6). In the same material we registered that 2% of the strains were resistant to doxycycline.

We also found a high frequency of resistance of CJC to ampicillin and penicillin G and the clinical use of these antibiotics has been associated with therapeutic failures. Ampicillin resistance of CJC is probably associated with beta-lactamase production in strains with MIC ≥ 64 (Wright and Knowles 1980). CJC was demonstrated to be resistant to most beta-lactam antibiotics.

Table 6. Susceptibility of CJC to some antimicrobial agents. MIC₉₀ (= drug concentration required to inhibit 90% of strains tested).

	Sweden ¹	Belgium ²	Belgium ³	Canada ⁴	USA ⁵	Indonesia ⁶
No of strains tested	100	95	86	133	11	28
Erythromycin	3.1	12.5	0.78	1.0	12.5	0.5
Tetracycline	1.6	1.52	1.56	16	0.8	≤0.25
Clindamycin	0.8	6.35	0.39	1.0	1.6	-
Nalidixic acid	32*	25	50	16	-	-
Gentamicin	1.6	1.56	0.195	≤0.5	0.8	≤0.25
Chloramphenicol	12.5	6.25	3.12	4.0	12.5	2.0

*Result obtained by disc diffusion test

¹Walder 1979 (III), ²Vanhoof et al. 1978, ³Vanhoof et al. 1980,

⁴Karmali et al. 1981b, ⁵Chow et al. 1978, ⁶Ringertz et al. 1981

Of parenteral drugs we consider gentamicin with 100% susceptibility as the first choice with chloramphenicol as a cheaper alternative but with a resistance of 2%.

Any change of the antibiotic susceptibility pattern can be detected if periodic surveys of MIC:s for CJC are performed.

Serotyping

Studies on the antigenic structure of campylobacter have for the most part been concerned with *C. fetus* ss *venerealis* which is a pathogen of cattle. Many of these studies were performed before 1973 when Butzler demonstrated that CJC was also an important pathogen in humans (King 1957, Winkelwerden 1966, White and Walsh 1970).

Using ovine and bovine strains, Berg et al. (1971) made a scheme of O-antigen serotypes demonstrated by agglutination. Bokkenheuser (1972) found that a passive haemagglutination test was more sensitive than agglutination but he did not use it for discriminating among CJC strains.

CJC, like *C. fetus*, probably possess OHK antigens with very complex and heterogen structures (McCoy 1975, 1976, Winter et al. 1978). Suspensions of CJC strains react differently according to the way in which they are prepared.

K-ag. Live suspensions probably reflect superficial heat labile antigens (capsular?) and are used in slide agglutination tests.

H-ag. Formalin treated bacteria show agglutination of a loosely flockular type suggesting flagellar antigen and are used in tube dilution tests.

O-ag. Heat treated bacteria probably represent somatic antigen, i.e. LPS (Dennis 1959, Ristic and Brandly 1959). Autoagglutination is common and therefore other tests must be used with this antigen e.g. CFT, fluorescent antibody test, indirect haemagglutination, ELISA, and passive haemagglutination tests.

Using live suspensions in slide agglutination Butzler made early serotyping experiments in 1974 with CJC without success and later Butzler and Skirrow 1978 recommended the use of formalinized bacteria for serotyping.

Penner and Hennessy (1980) described a useful serotyping scheme using crude unabsorbed antisera and passive haemagglutination with antigenic material extracted from CJC by heating bacterial suspensions at 100°C for 1 h and by EDTA treatment, procedures that are known to produce lipopolysaccharide, O -antigens, from gram-negative species (Neter et al. 1956). In 1981 Penner et al. were able to distinguish 44 serotypes when testing 819 strains, 8 serotypes were more common than the other and included 66% of the isolates. The number of untypable isolates was 6%.

Another serotyping scheme based on slide agglutination using live bacteria and specific absorbed antisera for the detection of heat-labile antigens, developed by Lior et al. (1982), recognized 21 serotypes. Of 615 human isolates, 86% were typable and 455 strains, agglutinated in 20 single antisera.

We have tested formalinized suspensions of 24 human strains in tube agglutination and ELISA tests against the corresponding 24 rabbit-immune sera (V). Antisera were prepared by injection with a mixture of harvested 48 hrs-culture suspension in Freund's adjuvant subcutaneously in four different places in a rabbit. In tube agglutination checkerboard titration did not reveal a distinct pattern of positive reactions but high titers

(>64) were found for all immune sera against the homologous strains and also occasionally positive titers (>8) against heterologous strains. No correlation could be seen between the titers and the results of the hippurate hydrolysis tests. In ELISA all 24 rabbit immune sera reacted against the homologous CJC strains.

Serology

Detection of antibodies to CJC is of diagnostic value in the diagnosis of extra-intestinal reactive complications such as arthritis and glomerulonephritis in patients with CJC infection. In early reports only autologous strains were used to detect antibody responses in patients after epidemic outbreaks (Butzler 1973, Skirrow 1977, Blaser et al. 1978).

With a combination of different reference strains and using more than one assay technique or antigen Watson and Kerr (1982) obtained positive reactions in 90% of patient sera and 14% of healthy controls and Kosunen et al. (1981) in 91% of patient sera and 4% of control sera. Moisman et al. (1981) with a complement fixation test found 90% of patient sera and 8% of control sera positive and Svedhem et al. (1982) with DIG-ELISA found that 90-100% of patient sera and 0-35% of healthy controls were positive, both using a pool of antigens.

Antibody determination (V) using OH-antigen in direct agglutination (Widal technique) showed no useful combination when 24 different CJC OH-antigens were tested in checkerboard titration against rabbit hyper-immune sera.

Bacterial LPS extracted by hot phenol water was then tested but the speci-

ficity in human sera tests using ELISA was low due to a nonspecific binding of gammaglobulin to LPS giving a high background.

Therefore we tested whole-cell antigen in ELISA (V) and found one formalinized strain that reacted with all 24 CJC hyper-immune antisera. The assay showed high sensitivity calculated to be 94%, the specificity was 95% and the effectivity 95%.

Analyzing sera from 67 CJC culture positive enteritis patients with campylobacter whole-cell IgG ELISA we detected significantly elevated antibodies in 49% of single patient sera and in 63% of paired sera and together in 82% of the patients tested. With addition of IgM analysis of the same 67 patient sera 77% of them were positive in IgM tests and together 94% of the 67 patients were positive in CJC, whole-cell ELISA when considering both IgG and IgM analyses, compared to only 5% of healthy controls.

IgG antibody formation against CJC occurs early in the course of infection and in enteritis patients elevated IgG and IgM titres could be detected during the first 10 days with peak concentrations between 11 days to 3 weeks after onset of infection.

In sera from children the lowest titres were detected between 0.5-3 years of age and in adults we normally found antibodies indicating a previous contact with campylobacter bacteria. Thus humans seem to be immunized and boosted by campylobacter probably by antigen found in chicken, meat or other food stuff (Grant et al. 1980, Stern 1981).

Pathogenic mechanisms

In our studies of both outbreaks and sporadic infections due to CJC, we found that the majority of affected persons were previously healthy which is in agreement with other reports (Blaser and Retter 1981).

After passage of the gastric acid barrier, the principal sites of infection for CJC seem to be jejunum, ileum and colon, with similar pathological features in each. At autopsy and laparotomy the jejunum and ileum has been found congested and haemorrhagic (King 1962, Evans and Dadswell 1967, Skirrow 1977, Blaser et al. 1980a). Furthermore, Cadranel (1973) isolated campylobacter from ileal and jejunal aspirates from children.

The passage of red blood in stools suggests involvement of the colon and this has been confirmed by sigmoideoscopy and colonoscopy. The microscopic examination of rectal biopsy specimens from patients with colitis have shown a histological picture indistinguishable from acute ulcerative colitis or Crohn's disease (Lambert et al. 1979, Blaser et al. 1980a, Duffy et al. 1980). In other cases the appearance of the biopsy sample has been similar to that produced by salmonella or shigella infections (Price et al. 1979).

The mechanisms by which CJC causes disease are not known. Invasion is likely to take place as ingested organisms can pass through the mucosa and invade the blood (VII) as is the case in e.g. salmonella (Takeuchi 1967) and yersinia infections (Pedersen et al. 1979).

Local invasion is another likely pathogenic mechanism but CJC has not yet

been identified in the mucosa of biopsies from human colon, perhaps because they appear in their coccoid resting form. Shigella is a typical example of a local invasive infection and some shigella strains also produce enterotoxin and cytotoxin which increase the severity of the lesions (Keusch and Jacewicz 1977). A similar quality can be assumed to exist in CJC which is further supported by the finding of pus and blood in many stool samples (Br Med J 1978) and could explain the great differences in symptomatology found in CJC enteritis patients.

Butzler and Skirrow (1979) demonstrated invasion of chicken embryo cells in 100% for five strains and invasion of chicken intestinal wall in all of 25 inoculated birds. Feecal leukocytes are seen in exsudative diarrhoea which is usually caused by invasive intestinal pathogens i.e. Salmonella, enteroinvasive E. coli (Harris et al. 1972) and Y. enterocolitica and there are also reports of feecal leukocytes in campylobacter associated diarrhoea (Mäki et al. 1979, Blaser et al. 1979).

In the same study Butzler found one hundred isolates negative for thermolabile enterotoxin (LT) activity (Butzler and Skirrow 1979) when tested with monolayer cultures of Y1 adrenal cells (Sack and Sack 1975). However, concerning the test for heat-stable enterotoxins (ST) Butzler found that with the infant mouse assay (Gianella 1976) 16 of the 100 isolates showed evidence of enterotoxin activity.

Experimental invasion can be demonstrated with HeLa cells (LaBrec et al. 1964). We have studied Yersinia and Shigella with this method and confirmed

their invasive capacity (Unpublished results). However, we have not been able to demonstrate the penetration of CJC into HeLa cells.

Tests for enterotoxin activity to reveal heat-labile toxin (LT) production have been performed with four of our strains by A-M Svennerholm (Unpublished results) using ganglioside immunosorbent assay (G_{M1} -ELISA) (Svennerholm and Holmgren 1978), ligated ileal loop of rabbit (Svennerholm 1975), and adrenal cell tests (Sack and Sack 1975), all with negative findings.

Using the infant mouse assay on our strains, T. Wadström (Unpublished results) found inconstant activity in some strains. Although virulence properties have not yet been allocated to plasmids found in CJC (Austen and Trust 1980) the bad reproducibility might be due to loss of a plasmid.

CLINICAL FEATURES

Major symptoms and signs

The symptoms and signs of a campylobacter infection are not characteristic enough to enable the physician to differentiate it from illnesses caused by other enteric pathogens. Mild forms of the disease resembling viral gastroenteritis are found and at the other end of the spectrum there are severe cases that resemble ulcerative colitis or Crohn's disease.

The incubation time is difficult to evaluate, as the source of infection is seldom known. Skirrow (1977) estimated the mean incubation time between 3-5 days with a range of 1.5 to 10 days. These data are in accordance with those reported by the two persons who voluntarily ingested known amounts of CJC and then developed symptoms on day 3-5 (Steele and McDermott 1978, Robinson 1981). In a report of 3 epidemic outbreaks in Malmö where the exact time for exposure was known (Billgren et al. 1982), we registered a mean incubation period of 2 days for the 14 adults and 3 days for the 4 children.

Our data on clinical features of CJC enteritis (VI) are based on records of patients whose symptoms were severe enough to make them see a physician.

In adult patients prodromes such as fever, malaise, headache, dizziness, backache and myalgia usually preceded the enteric symptoms.

(VI). Characteristically, abdominal pain also appeared before the début of diarrhoea and that was of great help to the physician in suspecting the

diagnosis. The pain at intervals, sometimes severe enough to resemble acute appendicitis, then accompanied the diarrhoea which after a day or two became watery with more than 10 evacuations per day. Reports of visible blood in stools among adult patients vary between 8% (Br Med J 1978) and 50% (McKendrick et al. 1982) and our study yielded 12%.

In our investigation (VI) the mean duration of symptoms was 4 days and most patients recovered in less than a week. However, about 20% had prolonged or severe illness and 11% needed hospital care, especially if they were elderly.

In children the symptoms are much the same as in adults (Table 7) but small children are less severely affected and many infants are afebrile (Butzler et al. 1973, Karmali and Fleming 1979b, Anders et al. 1981, Denneberg et al. 1982).

Table 7. Symptoms and signs in patients with CJC enteritis in per cent

	Adults				Children	
	Walder ¹ (Sweden)	Svedhem ² (Sweden)	Pitkänen ³ (Finland)	Blaser ⁴ (USA)	Karmali ⁵ (Canada)	Pai ⁶ (Canada)
Diarrhoea	88	83	98	100	95	100
Fever	75	61	80	67	86	77
Vomiting	9	8	41	25	30	35
Abdominal pain	84	24	48	96	60	67
Blood in stool	12	4	11	42	92	59

¹Walder 1982 (IV), ²Svedhem and Kaijser 1980, ³Pitkänen et al. 1981,

⁴Blaser et al. 1979a, ⁵Karmali and Fleming 1979b, ⁶Pai et al. 1979.

Dehydration was not a problem in our study of infants with enteritis (VIII) as vomiting, fever and watery diarrhoea were infrequent, being a more prominent feature in e.g. Rota virus infection. However, 3 of the 6 infants admitted to the hospital with CJC enteritis needed i.v. therapy. Diarrhoea with mucus, inflammatory cells and blood, either frank or diagnosed bio-chemically, are much more common in children than in adults (Mäki et al. 1979) which also was indicated in our study of enteritis in infants (VIII) where we found 2/6 CJC patients with overt blood in stools. Even higher figures have been reported by Karmali and Fleming (1979b) 90% and Anders (1981) 88%. In infants blood in stools is almost pathognomonic for CJC enteritis as bacillary dysentery and neoplasm are rare in our region.

From March through June 1979, the etiology of diarrhoea in 175 children from Ethiopia was studied (IX). CJC was isolated from 13% of enteritis patients and from 3% of matched controls. Most children with enteritis were between 6-12 months of age. This coincides with the weaning period and the source of infection is presumed to be contaminated food and water. The poor nutritional status among patients compared to controls can not be explained by different breast feeding habits only. The episode of diarrhoea bringing the child to hospital may have been one of many with successive impairment of the nutritional status.

Other symptoms and signs

Bacteremia is a constant feature in infection with *C. fetus* ss *fetus*, but this species rarely causes enteritis (Butzler 1978). In contrast bacteremia due to CJC is not often registered (Butzler et al. 1974, Guerrant et al.

1978). However, Levy in 1946 observed vibrio-like organisms in blood cultures from 13 enteritis patients when he sampled 39/151 patients with chills in an epidemic of 357 cases of enteritis. When bacteremia is diagnosed it seems to be selflimiting and sometimes even asymptomatic (Guerrant et al. 1978, Longfield et al. 1979, Eiden et al. 1980). However, some of the complications outside the gut which are seen in combination with CJC enteritis e.g. cholecystitis (Mertens and DeSmet 1979) and pneumonia (Pönkä and Kosunen 1982) are probably due to the haematogenous spread of the organisms.

So far we have only observed 5 cases of bacteremia due to CJC (VII). Four of our 5 patients had diarrhoea and all had fever and other signs of infection. The presence of bacteria in the blood stream suggests tissue invasion or tissue damage due to cytotoxic exotoxin. The frequency of CJC bacteremia is unknown as it is uncommon to make a blood culture from patients with enteric diseases. Furthermore, we found a correlation between a positive culture and blood samples taken early in the disease indicating an invasive phase at onset of illness.

Mesenteric adenitis and appendicitis have been reported in children and young adults (Skirrow 1977) and infectious proctitis has been shown in homosexual men (Quinn et al. 1980). Cases of post transfusional septicaemia (Peppersack et al. 1979), urinary tract infection (Davies and Penfold 1979), perimyocarditis (Pönkä et al. 1980), erythema nodosum (Lambert et al. 1982) and urticaria (Pitkänen et al, 1981) due to CJC infection have been reported. Convulsions in association with high fever may develop in young children with CJC enteritis (Havalad et al. 1980) and in adults frank meningitis has

been described (Wright 1979, Thomas et al. 1980, Norrby et al. 1980)

Sequele

Arthritis has been described in combination with CJC enteritis. One case of septic arthritis has been reported (Kowalec et al. 1980) but most cases concern reactive disease (Van de Putte et al. 1980, Kosunen et al. 1981). In these reports about half of the patients with reactive arthritis were found to possess histocompatibility antigen HLA-B 27. In our study (VI) one of 4 patients with post-infectious arthritis was assayed and had this specific tissue antigen.

Glomerulonephritis (Menck 1981) and hemolytic-uremic syndrome (HUS) (Chamovitz et al. 1981, Denneberg et al. 1982) are two other reactive complications with possible association to CJC enteritis. In HUS there is often a history of bloody diarrhoea before onset of the disease (Kaplan et al. 1976). Chamovitz et al. (1981) in a report of HUS in association with CJC isolated the bacteria in stool cultures from a patient with bloody diarrhoea.

We have reported a similar case of bloody diarrhoea in a patient with HUS in Malmö (Denneberg et al. 1982). In our patient we did not obtain faecal culture until 3 weeks after onset of illness and then it was negative. However, repeated ELISA serological tests of sera from the patient in convalescent phase showed a significant decrease in titer.

Laboratory features

Most patients have an elevated ESR and CRP and white cell blood counts

often show leukocytosis of 10-12.000 cells/mm³ but may be normal (McKendrick et al. 1982). Microscopic or chemical examination of stool specimens reveal higher frequencies of blood in stools than inspection. Polymorphonuclear leukocytes are seen in almost all stool samples (Mäki et al. 1979).

Differential diagnosis

Some of our patients with persistent high fever and frequent blood in stools were suspected of having an acute attack of inflammatory bowel disease (Unpublished results) and this has also been observed by others (Blaser et al. 1980a, Lambert et al. 1979). In infants with abdominal cramp and frank blood in stools intussusception is a differential diagnosis to remember.

Treatment

Like many other enteric infections CJC enteritis is frequently selflimiting (Blaser et al. 1979, Karmali and Fleming 1979a). In our study (VI) we found that only few patients (11%) required hospitalization and half of them recovered without any specific antimicrobial treatment. A short period of intravenous fluid treatment was required in the more severely ill patients, especially children (VIII), but most of them were able to take fluid orally. A premature return to ingestion of solid food resulted in the recurrence of symptoms.

Only 5% of our studied patients needed antimicrobial therapy because of life threatening disease. Nevertheless, when symptoms were prolonged, diarrhoea was frequent or bloody, high fever was present or when convalescent carrier had reactive complications, treatment with antimicrobial

agents seemed prudent. Treatment with antibiotics was also considered when eradication of CJC from stools of convalescent asymptomatic excretors among food handlers or hospital employees was desired.

Erythromycin has been recommended as the drug of choice for treatment of *Campylobacter* enteritis (Skirrow 1977). Of those given antimicrobial therapy (VI) treatment was performed with erythromycin in 56% and doxycycline in 26%.

For the rare systemic infections and bacteremias due to CJC, gentamicin and chloramphenicol have been used. Recent information suggests that parenteral treatment is not needed in all cases as selflimiting bacteremia due to CJC has been observed (Guerrant et al. 1978, Longfield et al. 1979, Eiden et al 1980).

We consider the value of antibiotics in the treatment of convalescent or asymptomatic excretors of CJC in relation to the extent to which such a patient constitutes a public health risk. Antibiotic treatment to prevent prolonged excretion of pathogens in stools can have the opposite effect as has been seen when treating intestinal salmonellosis or shigellosis with antimicrobials. This is however not the case with CJC. This has been demonstrated by controlled trials in Britain (Boyd and Murdoch 1979), USA (Anders et al. 1982) and Finland (Pitkänen et al. 1982)

In our study (VI), erythromycin or doxycycline treatment did not increase the duration of feecal excretion. In 22 convalescent excretors given either erythromycin or doxycycline 82% were negative in stool cultures within 48 h and CJC could not be recovered in stools from any of these patients after

conventional therapy and no relapses were recorded within 2 weeks after completed therapy.

EPIDEMIOLOGY

The epidemiology of *Campylobacter* enteritis is complex and a better understanding of the reservoirs and routes of transmission is needed to control CJC infections. There are parallels with salmonellosis in that the disease is a zoonosis, the organism is found in a wide variety of animals and the infection can be transmitted by the ingestion of contaminated food. There are also parallels with *Yersinia enterocolitica* infection since a high percentage of CJC enteritis patients are infected in Sweden.

Skirrow in 1977 reported that CJC was isolated in 7.1% of faeces samples from 803 unselected patients with diarrhoea, but in none of samples from controls. Lauwers and co-workers in Brussels (1978) reported an isolation rate of 1.3% of CJC in asymptomatic healthy persons, but they were newly arrived immigrants from North Africa living under poor conditions.

In study VI, covering 2.5 years, we found CJC incidence of 6.9% among 5,571 enteritis patients. The most common proven bacterial etiology of acute enteritis was CJC with almost as high incidence as salmonella, shigella, and yersinia together. When the study was extended over 4 years, the incidence increased to 8.3% in 8,991 enteritis patients (Table 8). Using the same isolation technique the incidence was only 0.25% among healthy controls.

In 6 of 95 children with enteritis admitted to hospital in Malmö, CJC was isolated from stools but in none of controls (VIII).

Table 8. Distribution of bacterial intestinal pathogens among enteritis patients in Malmö

	Isolation frequency (%)				
	1978	1979	1980	1981	4 years
Salmonella	4.9	3.2	3.9	3.8	4.0
Shigella	1.9	1.2	1.8	1.5	1.7
Yersinia enterocolitis	2.3	2.3	2.4	2.9	2.5
Campylobacter	6.2	7.8	10.1	10.5	8.3
Total	15.3	13.4	18.1	18.8	16.3

However, the prevalence of CJC infection in some of the developing countries is probably much greater than in the industrialized countries. In studies in South Africa (Bokkenheuser et al. 1979) and Bangladesh (Blaser et al. 1980d), 16-35% of hospitalized children under 2 years of age with diarrhoea excreted CJC and controls excreted the bacteria in 16-39%. In our study in Ethiopia (IX) 13% of the children under 2 years of age with diarrhoea excreted CJC compared with 3% for matched controls. The high percentage of excretion among healthy children makes the interpretation of isolations from children with diarrhoea difficult in developing countries.

Seasonal distribution

Most patients with CJC enteritis are infected during the warm months of the year, e.g. in Belgium (Lauwers et al. 1978), England (Weekly Epidemiol Rec 1979) and USA (Blaser et al. 1979).

In our study in Malmö (VI), 2/3 of the patients were infected during the summer months. The peak incidence occurred from July to September (Fig. 2).

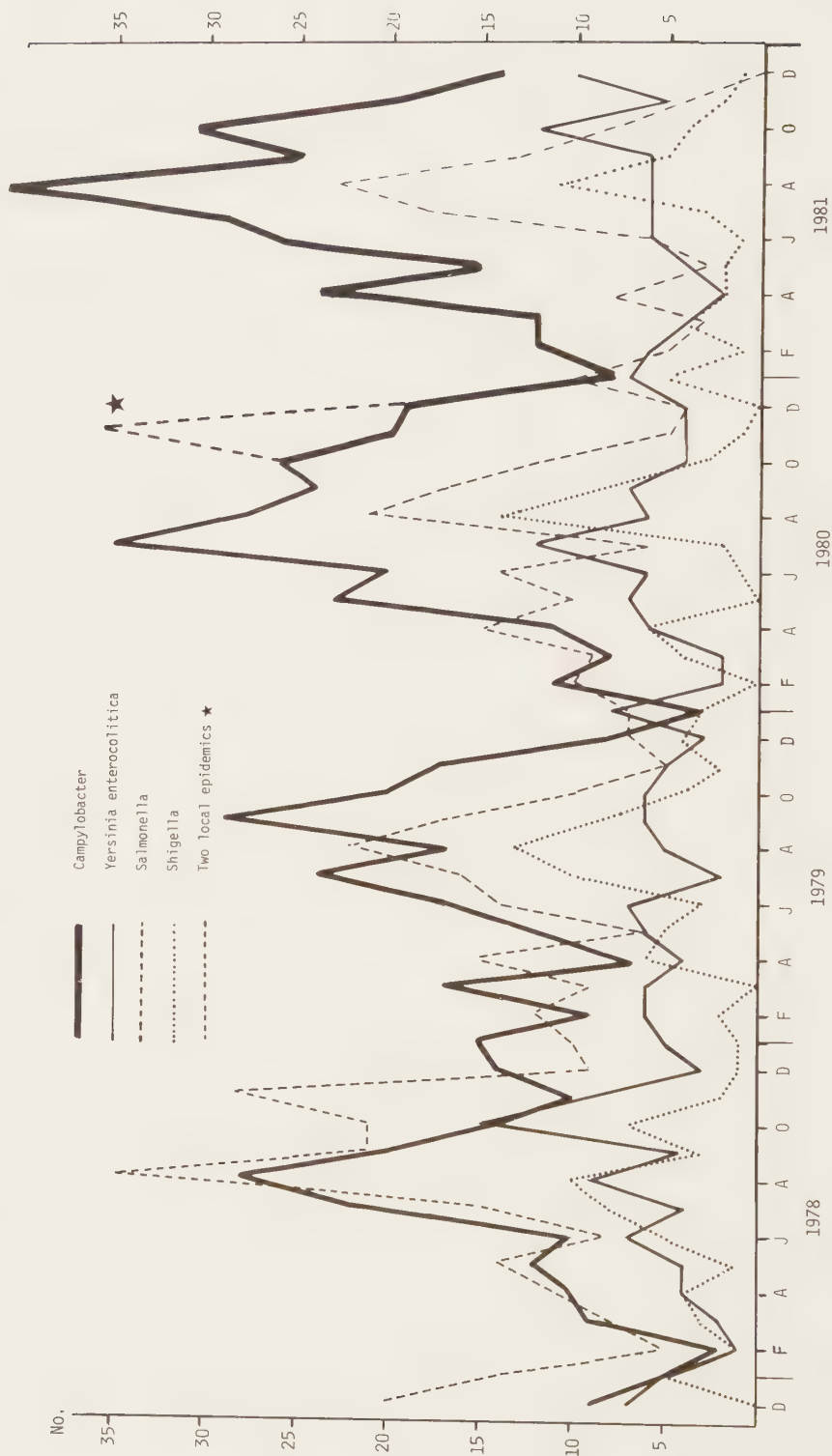
In developing countries, the isolation frequency of CJC from patients with diarrhoea was much the same in the rainy season as in the dry season (De Mol and Bosmans 1978, Bokkenheuser et al. 1979, Blaser et al. 1980d). Our Ethiopian study (IX) was performed during the rainy season.

Geographical distribution

The geographical distribution of infections was determined by examining the records of patients with CJC enteritis (VI) attending the Department of Infectious Diseases, Malmö General Hospital. Fifty-three per cent of these patients had acquired their infection in Sweden and most of them lived in the urban area of Malmö in the south part of Sweden. During the study period there was an equal distribution over the months between domestic and imported cases with extremes of 76% for domestic and 67% for imported cases. No obvious pattern was seen during the 3 years - 1978, 1979 and 1980 (Fig. 3).

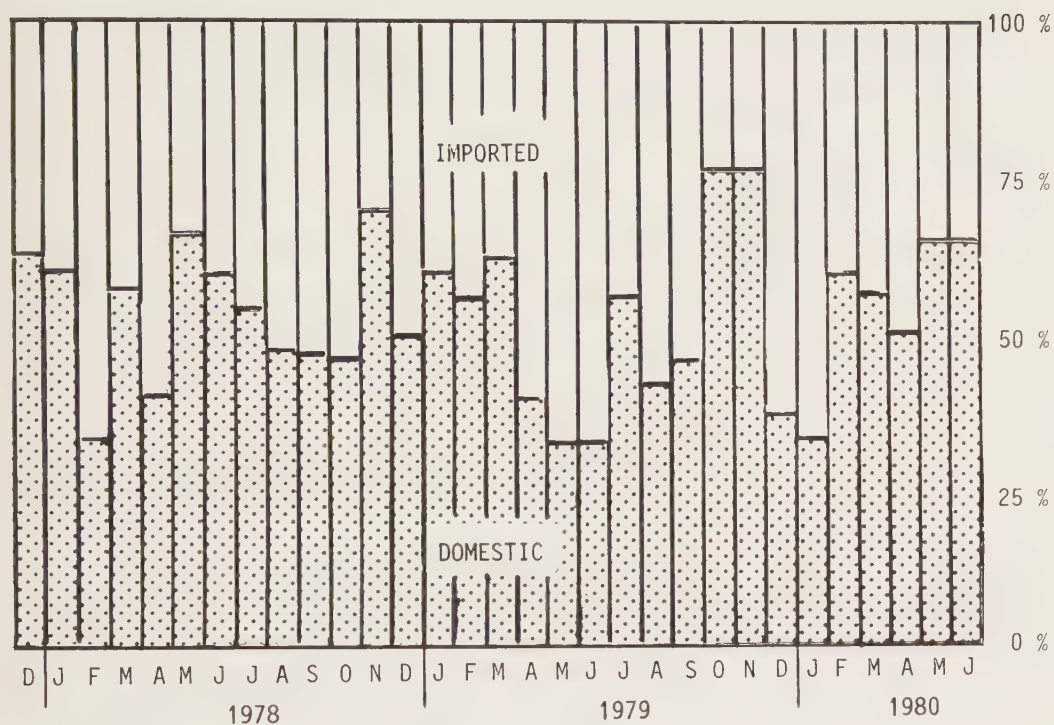
A recent journey (within 2 weeks) outside Scandinavia was recorded for 47% of all the patients studied. Of the patients between 21 and 45 years 56% had acquired their infection in non scandinavian countries. The highest frequency of infection was noted for travellers to African-Mediterranean countries and to Asia.

Number of patients in Malmö with bacterial intestinal pathogens during 4 years.



Figur 2

Percentage of campylobacter cases contracted in Sweden and imported ones during 31 months.



Figur 3

A study from western Sweden (Svedhem and Kaijser 1980) showed that the proportion of patients infected outside Sweden was 73%. Their study also revealed an isolation frequency for CJC of 11% in enteritis patients. These higher figures found in Gothenburg are probably due to a higher frequency of travelling by charter flights from western Sweden in summer time (Travel agencies, personal communication). This assumption is further supported by the fact, that almost 1/3 of the imported cases in that study, versus 1/4 in our study came from the European-Mediterranean area where the most popular

resorts for Swedes are situated. In a survey of campylobacter infections in Britain 1977 compiled by CDSC, it was found that travelling to foreign countries was not associated with CJC enteritis, though a recent journey abroad was mentioned in 1/5 of the cases.

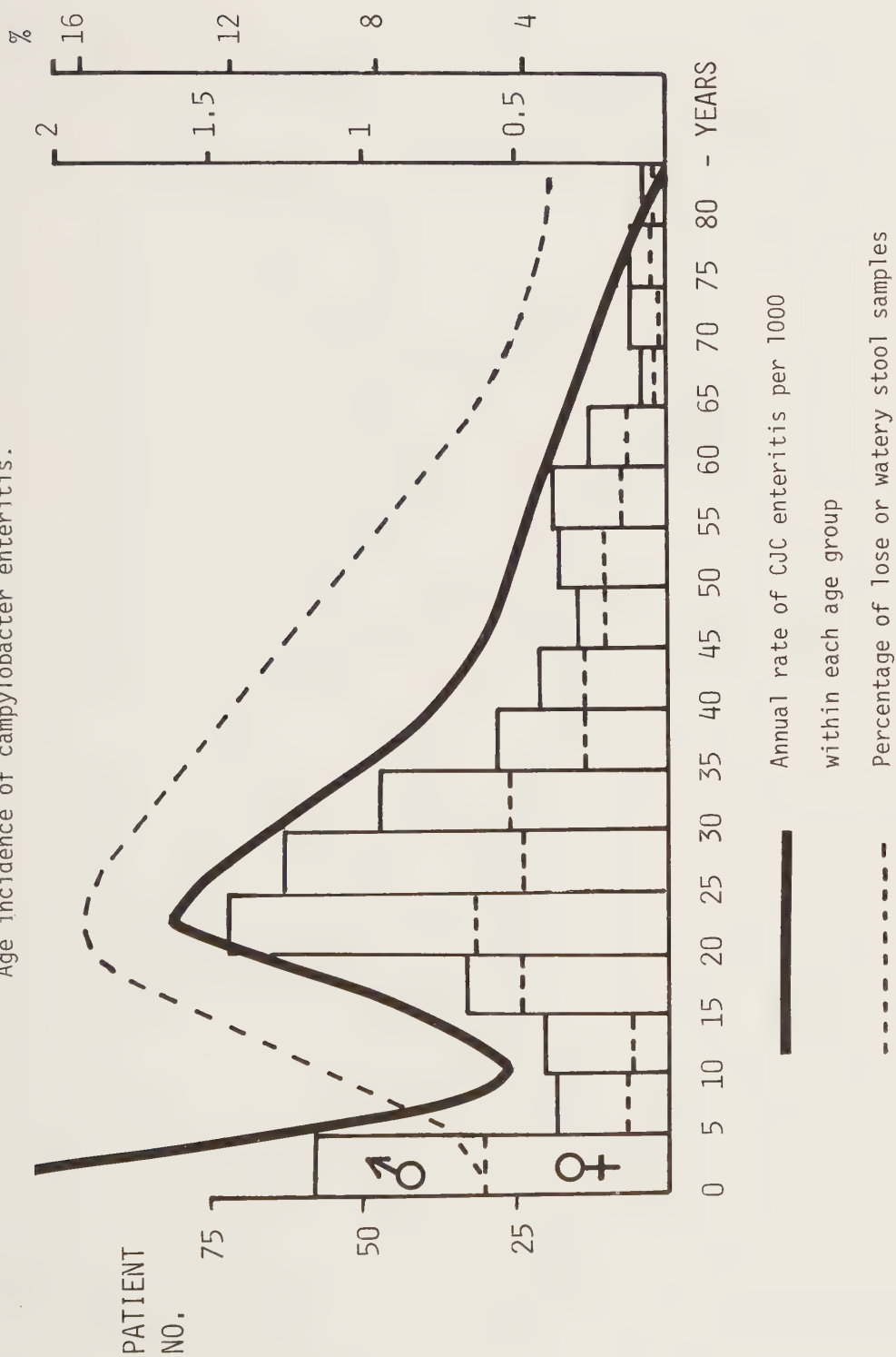
Age and sex distribution

In other Swedish studies about 50% of the patients were between 16 and 35 years (Bengtsson and Uhno 1978, Falsen et al. 1978, Henriksson et al. 1978). Our figures in study VI 49% with a mean age of 27.5 years, are in accordance with their results.

It is difficult to measure the true incidence of infection in different age groups. The most accurate presentation would be by the percentage of positive faecal samples within each age group, even if there is a disproportion in faeces samples submitted for culture. However, there is an over-sampling from patients younger than 5 years relative to adults. Of the CJC patients in our study 13% were children below 5 years of age. This relatively high figure is probably a reflection of the high frequency of diarrhoeal disease in this age group and not an increase in the relative occurrence of CJC enteritis compared to other causes of enteritis. Another age distribution is shown in figure 4 with the percentage of faeces samples which contained CJC within each age group.

On the whole Campylobacter enteritis is more or less equally common in both sexes. However, a slight predominance of boys below the age of 15 and of women over the age of 40 was found.

Age incidence of campylobacter enteritis.



Figur 4

Curves represent best fitting lines.

Sources of infection

Bacteria indistinguishable by present available criteria from strains of CJC that affect humans have been isolated from many mammalian and avian species (Smibert 1978) but we do not yet know how often human infections are derived from animals.

In animal studies CJC has been isolated from e.g. healthy swine (Svedhem and Kaijser 1981), cattle (Smibert 1978, Svedhem and Kaijser 1981), sheep (Smibert 1978, Svedhem and Kaijser 1981), horses (Atherton and Ricketts 1980), goats (Dobbs and McIntyre 1951), rodents (Smibert 1978), hamsters (Fox et al. 1981), monkeys (Tribe and Frank 1980) and cats (Bruce and Ferguson 1980). Infection with CJC may cause diarrhoeal illness in calves, lambs (Firehammar and Myers 1981), monkeys (Tribe and Frank 1980) and swine (Ferne et al. 1975).

Porcine isolates are solely hippurate negative *C.coli* strains (II), but they contribute only to 13% of human strains. Thus pig seem to be a minor source of infection for man.

Chickens were first suspected of being sources of campylobacter enteritis in humans by King (1957, 1962), who found that strains of "related vibrios" from cases of avian infectious hepatitis were indistinguishable from human strains. Excretion of CJC in adult birds is not associated with illness (Blaser and Reller 1981). Cross-contamination of poultry carcasses easily occurs during processing which means that many birds sold over the counter, both deep frozen and fresh, are contaminated with campylobacter (Skirrow 1977, Bruce et al. 1977, Grant et al. 1980). However, correct

methods of preparation will prevent the spread of organisms to other food stuff and proper cooking will kill them, but Hayek 1977 suggested that cross-contamination took place between uncooked and cooked food. Moreover, there is so far no evidence that CJC multiplies readily in or on food.

In Malmö we have observed 2 small outbreaks in schools where the same cutting-boards were used for preparing first a chicken and then a salad without cleansing them in between (Billgren et al. 1982). Broiler chicken seems to be the major source of infection for disease in man. Eight of 22 (36%) hen isolates and none of 17 chicken isolates (II) were hippurate negative compared to 13% of human strains, but all poultry isolates were genetically similar to human CJC strains. However, the 17 Malmö chicken strains initially grew only at 42°C, but after subculturing they successively adopted to grow at 37°C. Thus culturing temperature could be a serious source of error in epidemiological studies of chickens.

There is increasing epidemiological evidence that campylobacter enteritis affects dogs (Wheeler and Borchers 1961, Skirrow 1977, Lindqvist et al. 1978) and cats (Blaser et al. 1982), especially puppies and kittens (Hastings 1978, Blaser et al. 1980b). Experimental infections in pets have so far been unsuccessful (Prescott and Karmali 1978 , Unpublished results).

Many authors imply that strains of campylobacter isolated from dogs are identical to CJC (Blaser et al. 1978). However, when analyzing 98 isolates from dogs both with and without diarrhoea (I) we found that 2/3 of the strains had very weak or absent catalase activity (CNW). They all grew on Skirrow's medium and phenotypically they were different to human CJC strains,

genetically they formed a separate group. Moreover 91/98 strains were hippurate negative. Among human isolates we have seen no CNW strains and we only found 13% of human strains negative in hippurate test. Thus, only few human isolates seem to come from dogs.

Commercial pork, lamb and beef carcasses (Hayek 1977, Stern 1981) may be contaminated with CJC. A high percentage of minced meat (Svedhem et al. 1981) has been reported to be contaminated, but in an extensive British survey of 4,933 retail samples, CJC was only isolated in 1% of them (Turnbull 1982) and we have not found CJC in any of 50 minced meat samples cultured (Unpublished results).

Both salt (Pearsson et al. 1977) and fresh water (Knill et al. 1978) have been shown to be contaminated with CJC. Two large outbreaks have been attributed to the introduction of surface water into the fresh water supply system (Tiehan and Vogt 1978, Mentzing 1981). Non purified water is a common vehicle for the transmission of enteric infections and a study suggested that CJC survived longer at 4°C than at 25°C (Blaser et al. 1980c) indicating that cold water may be a more effective vehicle for CJC contamination.

Cattle frequently carry CJC in their intestine (Smibert 1978) and an outbreak of campylobacter enteritis due to the consumption of unpasteurized milk was reported already in 1946 by Levy. Since 1978 milk has been implicated in at least 13 documented outbreaks of campylobacter infection of substantial size in Britain (Robinson et al. 1979, Robinson and Jones 1981, Porter and Reid 1980, Jones and Willis 1981) and 2 have been reported from USA (Taylor et al

1979; Blaser et al. 1979). The mechanism of transmission is most likely direct faecal contamination during or after milking. Pasteurization, if correctly carried out, will effectively eliminate campylobacter from milk. However, in England and Wales 3% of milk retailed is still unpasteurized (Robinson and Jones 1981).

Spread of the infection via person to person contact has been described but exclusively from infants and small children who were incontinent of faeces to their tenders (Blaser et al. 1981) e.g. in day-nurseries (Cadranel et al. 1973, Itoh et al. 1980).

In 3 minor epidemic outbreaks in Malmö with 18 cases of CJC enteritis we obtained epidemiological information about all persons within the same households as the index cases (Billgren et al. 1982). All household contacts were asymptomatic and none of those cultured shed any organisms.

Vertical transmission of CJC from symptomatic or asymptomatic mothers to their neonates has been reported (Mawer and Smith 1979, Karmali and Tan 1980, Vesikari et al. 1981) but has not yet been registered in Malmö.

In the private food industry in England food handlers are not allowed to work during the period they excrete the organism in their stool (Jones and Harro 1981).

No secondary transmission from adults has been observed in Malmö (Unpublished results), nor has asymptomatic food handlers or hospital personnel been implicated as sources of infection, but we believe that no employee should work with diarrhoea and that asymptomatic convalescent excretors with the

jobs mentioned above, should only be allowed to go back to work after thorough instructions of hand washing routines. These procedures are the same that Swedish health authorities prescribe after salmonella infection.

In conclusion we believe that ingestion of infected or contaminated food, such as poultry and milk, probably accounts for most cases of CJC infection in humans and only a minor part is thus due to ingestion of pork, close contact with domestic animals and pets or spreading from person to person.

Dose of infection

There is some controversy about the dose of infection for CJC. Gastric acid is a potent barrier against ingested pathogens and an important determinant of infectious doses. Blaser et al. (1980c) have shown that solutions with pH <3.0 adversely affected the survival of CJC but solutions with pH >3.6 had virtually no effect on survival. These data suggest that CJC infection may result from:

1. Ingestion of large numbers of organisms.
2. Ingestion of small numbers of organisms in buffer, such as milk, which is an excellent buffer.
3. Few organisms in connection with rapid gastric wash through as e.g. with water intake on an empty stomach.

Three persons reported in the literature have ingested known amounts of bacteria. In 1977 McDermott in Australia ingested 10^6 campylobacter and developed abdominal discomfort 3 days later and diarrhoea on the 5th day (Steele and McDermott 1978).

In 1981 D.A. Robinson, Manchester, England, swallowed 500 organisms in 180 ml milk and CJC was cultured from faeces on day 2 and diarrhoea with

abdominal cramp developed on day 4 (Robinson 1981).

In an experiment with dogs J.F. Prescott accidentally was exposed to less than 2.5×10^{10} bacteria (Prescott and Karmali 1978). He developed mild diarrhoea on day 5 and 6, whereas none of the puppies and kittens given a much higher dose, showed any sign of disease.

Attack rate

Attack rate of an infectious disease is dependent on the virulence of the micro-organism and the immunity in the population. Attack rates of some major epidemic outbreaks of CJC enteritis have been reported. In a milk-borne outbreak in Aberdeen involving 616 persons (Porter and Reid 1980) the attack rate was estimated to be 50%. In a water-borne outbreak reported by Mentzing (1981) from Sweden 10-30% of those exposed fell ill, and in another outbreak caused by water that was reported in Vermont, USA, 14.4-23% of the population at risk developed diarrhoea (Tiehan and Vogt 1978).

In our own experience from Malmö we know that ingestion of bacteria in infectious doses not necessarily causes clinical disease but sometimes gives asymptomatic excretion (Unpublished results). Accordingly we found 0.25% asymptomatic carriers among healthy controls in Sweden (VI) and 3% in Ethiopian children (IX). Moreover, in one epidemic at a day care unit in Malmö (Billgren et al. 1982) 7 children and 4 adults were exposed to the same source of infection. Four of the children and all 4 adults developed diarrhoea, but CJC was cultured from stools of all 7 children and all 4 adults. Thus about 1/3 of those exposed were asymptomatic excretors.

Similar figures have been reported from a milk-borne outbreak where 20-25% of the persons that developed antibodies were asymptomatic (Jones et al. 1981).

It is well known from developing countries that children are often asymptomatic excretors of CJC, e.g. 16% in a South African ghetto (Bokkenheuser et al. 1979) and 17.7% in a Bangladesh study (Blaser et al. 1980d). However, in spite of optimal isolation technique we only found 3% of healthy controls in Ethiopian children excreting CJC and De Mol (1978) none in Central Africa.

Nevertheless, this may indicate that subinfectious doses of CJC may cause a transient episode of asymptomatic excretion of CJC in stools and that the time of excretion in "compromised children", e.g. in developing countries may be prolonged.

In conclusion, our family studies indicated that infectivity was low probably because of the short survival of these organisms in dry state outside the body. These facts can explain the few secondary cases reported, in spite of the low dose of infection - about 500 organisms postulated by Robinson (1981).

Persistence of the organisms in stools

There is always a period of convalescent excretion of CJC in stools after diarrhoea has stopped (Pai et al. 1979, Karmali and Fleming 1979, Porter and Reid 1980).

Our study (VI) showed that for most patients disappearance of the organism from stools is rapid, 80% being culture negative within one month, but some are carriers for many months.

As mentioned above, it is not known whether these convalescent and asymptomatic excretors of CJC constitute a public health risk or not.

Surveillance

The epidemiological tracing of a common source in epidemic outbreaks by serotyping or by biochemical typing is very important and also in diagnosing secondary cases. In Canada, Penner et al. (1981) has found 44 different serotypes in a collection of 819 isolates obtained from humans and animals but 8 serotypes accounted for 66% of the isolates and only 6% of the isolates were untypable.

In outbreaks it has been shown that more than one serotype or biotype can be present (Abbot et al. 1980, Jones et al. 1980) but usually it is convenient to show antibody response in humans by passive agglutination against homologous strains.

We have studied some biochemical reactions of CJC in order to find an epidemiological marker and found that sensitivity to Mercury could be of epidemic value since about 15% of CJC strains from humans in Malmö were resistant (Unpublished results).

GENERAL SUMMARY

- *Campylobacter jejuni* and *C. coli*, in this study together designated CJC, were separated by the hippurate hydrolysis reaction. Of human CJC isolates *C. jejuni* constituted 87% and *C. coli* 13%. All broiler strains, 64% of poultry strains but none of porcine isolates were *C. jejuni*. Thus pig isolates are a minor source of infection for man.

C. jejuni and *C. coli* formed separate homogenous DNA homology groups, providing further evidence for the validity of hippurate hydrolysis as differentiating test between the species.

- Two thirds of CJC isolates from dogs had a negative or weak catalase reaction (CNW) and they were genetically different from all other campylobacter strains including human isolates. Nalidixic acid resistant strains of *C. coli*, NARTC-like strains, composed a genetically heterogeneous group separated from other *C. coli* strains, whereas nalidixic acid resistant strains of *C. jejuni* were genetically similar to other *C. jejuni* strains.
- Antibody response to CJC infection was investigated using an enzyme-linked immunosorbent assay, ELISA. With a mixture of lipopolysaccharide (LPS) from two CJC strains as antigen in ELISA, non-specific binding of human immunoglobulin to the LPS antigen was obtained, and antibody response was detected in only 70% of CJC enteritis patients. One of 24 formalinized whole CJC bacteria reacted with all corresponding rabbit antisera and essentially no unspecific binding was recorded.

Analyzing immune response with campylobacter whole-cell IgG and IgM ELISA 94% of patients with CJC enteritis were positive compared to only 5% healthy controls. Children had low antibody titres especially before 3 years of age, but in adults we normally found antibodies probably due to immunization and boosting by campylobacter antigens found in food stuff.

- Doxycycline and gentamicin were the most active drugs inhibiting all 100 CJC strains at concentrations achievable in serum when tested by an agar dilution technique. In a disc diffusion test of 435 strains using Swedish breakpoints, 2.0% of CJC strains were resistant to doxycycline and chloramphenicol, 2.6% to erythromycin but none to gentamicin. All strains were resistant to essentially all beta-lactam antibiotics tested. In 27 (13%) enteritis patients with CJC in stools antibiotic treatment was instituted. Chemotherapy was given either as erythromycin (15) or doxycycline (7) to 22 of the patients. Within 2 weeks after therapy CJC could not be recovered in stools from any of these patients.
- Five cases of bacteremia with CJC were investigated, 4 of the 5 patients had diarrhoea. Invasion of the blood is rarely reported in combination with CJC infection. The accumulation of our cases may be explained from the short duration between onset of high fever and obtaining blood cultures, and the use of blind subcultures in the laboratory. Invasion of the blood may be one of the pathogenic properties of CJC.

- In the study during 4 years CJC was the most common proven bacterial etiology of acute enteritis in Malmö. The incidence for CJC in 8991 enteritis patients was 8.3%, only 5 (0.25%) of 2000 healthcontrols had CJC in their stools. Half of the patients were between 16-35 years, 53% had acquired their infection in Sweden. The most common symptoms were high fever in 75%, frequent watery diarrhoea in 45% and colics or abdominal pains in 84%. Uncommon signs were blood in stools 12% and reactive non-septic arthritis 2%. *Campylobacter* enteritis was in most cases not a severe disease and only 11% were admitted to hospital with a mean nursing time of 4 days. More than 80% of the patients were permanently negative in stool cultures within one month.
- The isolation rate of CJC in Swedish infants with enteritis was 6% and no isolates were made among control children. CJC was found in 13% of enteritis children in Ethiopia and 3% of controls. Children were less severely affected compared to adults and many infants were afebrile. In Swedish infants we found 2/6 enteritis children with overt blood in stools.

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Appendix

THERMOTOLERANT CAMPYLOBACTER WITH NO OR WEAK CATALASE ACTIVITY ISOLATED FROM DOGS

Karin Sandstedt. †, Jan Ursing‡ and Mats Walder‡

†National Veterinary Institute, Ultuna, S-750 07 Uppsala, Sweden

‡Department of Medical Microbiology, University of Lund,
Malmö General Hospital, S-214 01 Malmö, Sweden

ABSTRACT

Thermotolerant Campylobacter strains isolated from dog feces were characterized by phenotypical tests, DNA base composition and DNA-DNA-hybridization. Out of 98 strains 63 were catalase negative or weakly reacting (CNW); they were found in diarrheic as well as in healthy dogs. The CNW strains were all nalidixic acid sensitive, hippurate negative and grew at 42°C but not at 25°C. Seven strains were further investigated. They were sensitive to 2,3,5-triphenyl-tetrazolium chloride, reduced nitrate and produced H₂S in the lead acetate test but not in triple-sugar-iron agar. The mol% G + C for five CNW isolates ranged from 35.2 - 35.8 which is higher than reported for any thermotolerant Campylobacter species. The strains also formed a well delimited DNA homology group with 80 % or more intragroup relatedness and about 40 % related to C. coli and C. jejuni.

INTRODUCTION

Dogs, both with and without enteritis, may harbor thermotolerant Campylobacter. The frequency of Campylobacter carriers among diarrheic dogs has variably been reported to be between 1 and 75 per cent (11, 12, 9, 5, 21). Thus, there could be a possibility of transmission of the bacteria from dog to man (23), and in searching for sources of human Campylobacter diarrhea (8, 6) fecal samples from dogs should be considered. In articles concerning Campylobacter carriers among dogs, it has been claimed that these strains are identical to C. jejuni or C. coli (18, 1, 2, 23). However, in this communication we present a collection of canine fecal Campylobacter, in which the majority of strains belong to a hitherto undescribed phenotypically and genetically defined group.

MATERIALS AND METHODS

BACTERIAL STRAINS. Within a 2-year period, 98 strains of thermotolerant Campylobacter were isolated from dogs, 79 of which had diarrhea and 19 of which appeared healthy. Some strains were isolated during an epizootic of canine parvovirus (22). The fecal samples were collected at an animal clinic. For comparison was used a material of 900 human fecal strains isolated during 4½ years at Malmö General Hospital. Campylobacter type strains were received from Collection de l'Institut Pasteur, Paris; they are listed in Table 1. Strain Escherichia coli B (NCTC 10537) was obtained from National Collection of Type Cultures, London and was used as reference strain for the DNA work.

ISOLATION METHOD. Every fecal sample was suspended in 5 ml Bactothiol broth (Oxoid). After one hour visible particles had sedimented and motile bacteria had been given time to disperse. At that time, the upper 2 ml of the broth were removed by a syringe and passed through a 0.65 µm Millipore filter on a bovine blood agar plate without antibiotics. The plates were incubated under microaerophilic conditions at 37°C for 72 h, before examination. The samples were also analyzed for Salmonella and Yersinia. Some of the animals were serologically tested for antibodies against canine parvovirus. Campylobacter strains from human cases of enteritis were isolated as previously described (29).

PHENOTYPIC CHARACTERIZATION. All cultures were incubated under microaerophilic conditions for 48 h at 37°C unless specified. Temperature tolerance was tested on blood agar at 25, 37 and 42°C. Strains were also tested for growth on blood agar with selective supplement according to Skirrow (Oxoid Ltd., London, England) at 42°C. Capacity to grow in the presence of 2,3,5-triphenyl-tetrazolium-chloride (TTC) was demonstrated on blood agar containing 400 µg TTC/ml. Susceptibility to nalidixic acid was tested with 30 µg discs (Biodisk, Solna, Sweden) on blood agar plates; strains forming an inhibition zone of 10 mm or more were considered sensitive. Motility was studied in hanging drop preparation. For the catalase test, bacterial growth

from a blood agar plate was transferred with a plastic loop to a drop of 20 % hydrogen peroxide on a slide and observed for 5 min. If bubbles appeared within 10 sec the reaction was considered positive, after that weakly positive. The oxidase test was performed according to Kovács (14). Production of H_2S was tested with a lead acetate strip over a Bactothiol broth culture as well as on TSI agar (BBL Microbiology Systems, Cockeysville, Md.). Hippurate hydrolysis was tested according to Hwang & Ederer (13). Nitrate reductase was demonstrated in Diagnostic Thioglycollate broth (BBL) supplemented with 0.1 % KNO_3 and with reagent according to Cowan (7).

ISOLATION OF DNA. The method described by Brenner et al. (3) was used with a few modifications. The cells were harvested from blood agar and lysed for 10 min at $37^{\circ}C$. The final preparation was dissolved in 0.1 SSC (SSC = 0.15 M NaCl + 0.015 M Na_3 -citrate, pH 7.0) and stored at $+4^{\circ}C$.

DETERMINATION OF BASE COMPOSITION. Mol% G + C was determined by thermal denaturation using Gilford 250 spectrophotometer equipped with 2527 thermo-programmer. The DNA was diluted to 50 $\mu g/ml$ in 0.1 SSC. The DNA solutions were heated in microcuvettes (0.30 ml) with 1 cm light path at a rate of $0.5^{\circ}C/min$ starting at $55^{\circ}C$. Four DNA:s could be melted simultaneously; Escherichia coli B DNA was included in every run. The thermal denaturation midpoint (T_m) was calculated graphically. The mol% G + C was computed using the formula of Mandel (19) assuming the mol% G + C of E. coli B to be 52.0 (20). Four T_m -determinations were performed on each DNA.

DNA HYBRIDIZATION. DNA solutions were disrupted by sonication to a fragment length of 600-800 nucleotides and heat-denatured. Labelling was performed in vitro with ^{125}I using the method of Tereba & McCarthy (27) as modified by John L. Johnson (pers. comm.). The reaction volume was 100 μl containing 10 μg DNA, 100 $\mu Ci Na^{125}I$ (IMS. 30, Amersham International, Amersham, England), 7×10^{-6} M KI, 4×10^{-4} M $TlCl_3$ and 0.05 M Na-acetate buffer pH 4.8. After termination of the reaction, heating to $60^{\circ}C$ during 30 min was performed twice, each time followed by separation on a prepacked G-25 Sephadex column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.1 SSC. Seventy-five μg unlabelled DNA was reassociated with 0.02-0.03 μg labelled in 0.5 ml phosphat buffer containing 0.42 M K^+ for 16 h at $60^{\circ}C$ (considered

as optimal temperature) or 75°C. Elution of single-stranded and double-stranded DNA on hydroxy-apatite followed the batch procedure of Brenner et al. (4). For the thermal elution profiles the temperature increments were 5°C. The eluates were counted in a gamma counter (1270 Rack-Gamma, Wallac, Turku, Finland). The elutions were performed in duplicate. Relative binding ratio (RBR) of heterologous DNA was calculated as percentage of homologous binding.

RESULTS

All 98 canine isolates were microaerophilic, gram negative, motile, curved rods with positive oxidase reaction. They all grew at 42°C but not at 25°C. The catalase reaction was positive for 35 of the strains but negative or weak (CNW) for the other 63. This difference between the groups was clear-cut as the positive reactions appeared very rapidly and the weakly positive came later than 30 seconds; however, the outcome could vary between weak positive and negative when different subcultures of the same strain were tested. The fraction of strains isolated from healthy animals was the same for the catalase positive strains as for the CNW phenotype. All Campylobacter strains recovered from human fecal samples were catalase positive.

Of the 35 catalase positive strains, 7 hydrolyzed hippurate and one (a hippurate negative strain) was resistant to nalidixic acid. All 63 CNW strains were hippurate negative. Seven CNW cultures and 2 hippurate negative, catalase positive cultures were further investigated. Characteristic properties of these isolates and of the reference strains are shown in Table 1. All 98 canine isolates grew well on Skirrow's selective medium at 42°C.

Genetic data appear in Table 2. Mol% G + C was estimated for 5 CNW strains (35.2 - 35.8), two catalase positive, hippurate negative strains (32.3 - 33.1) and for the type strains (31.0 - 34.3). Strain C 303 was selected for radioisotope labelling because it gave the best yield of DNA. The specific activity was estimated to about 1.5×10^6 cpm/ μ g DNA. The RBRs at 60°C ranged from 80-96% for the CNW strains and from 38-40 for the other thermotolerant strains. The relatedness to the type strains of C. fetus subsp. fetus and C. sputorum subsp. bubulus was 17% and 19% respectively. There was

no significant binding with E. coli B. The values for $\Delta T_{m(e)}$ and RBR at 75°C agreed with the binding rates at optimal temperature.

In one of the dogs Salmonella poona was found, whereas Yersinia spp. were not found at all. Twenty-three animals were infected with canine parvovirus according to serological examinations.

DISCUSSION

PHENOTYPIC PROPERTIES. The general characteristics of the canine isolates undoubtedly place them in genus Campylobacter (25). Two thermotolerant Campylobacter species, that are unable to grow at 25°C, are presently recognized: C. coli and C. jejuni. Since the introduction of the hippurate test for characterization of Campylobacter (10), there is increasing evidence (16) that the test is reliable for differentiating these two species. Thus, of the catalase positive isolates 7 conform to C. jejuni and 28 to C. coli. The nalidixic acid resistant strain of C. coli could possibly belong to the NARTC phenotype of Skirrow (24); its properties were, however, not further investigated. Not considering the catalase test, the CNW strains differed from C. coli only in lack of tolerance to TTC; however, TTC sensitive strains of C. coli do occur (Walder et al. Unpublished results).

GENETIC PROPERTIES. The mol% G + C of the type strains were in agreement with the published results (20, 28). The G + C content of the canine isolates resembling C. coli was similar to that of the type strain. The CNW isolates formed a genetically separate group with G + C content more than 3% higher than for any other thermotolerant strain and a DNA relatedness to C. coli and C. jejuni of about 40%, falling to insignificant levels at 75°C. The binding rates against C. fetus subsp. fetus and C. sputorum subsp. bubulus were considerably lower than against the thermotolerant species but they nevertheless indicate some degree of genetic relationship. The homogeneity of the CNW group was reflected by very similar mol% G + C and high DNA-relatedness also at 75°C and correspondingly low differences in thermal elution midpoint (less than 3.0).

TAXONOMIC ASPECTS. Genus Campylobacter is usually divided in one catalase positive and one catalase negative group, the latter consisting of one recognized species, C. sputorum. To our knowledge, catalase negative, thermotolerant Campylobacter have not been reported. Tanner et al. (26) described the catalase negative C. concisus with G + C content of 34-38%. The similarly catalase negative C. sputorum subsp. mucosalis was shown by Owen & Leaper (20) to have a base composition of 37.8% G + C. Strains of these species have not been available to us during this study. However, neither of these bacteria have been reported to be thermotolerant; moreover, C. concisus was described as nalidixic acid resistant (26) and C. sputorum subsp. mucosalis as very strongly H₂S positive (15). Thus it does not seem probable that the CNW group is identical to any of these species. Although the CNW isolates seem to make up a good genospecies, we refrain for the present from proposing a species designation as we feel that the phenotypic differentiation should rely on more than one property. Strain C 231 is suggested as reference strain for the CNW group.

ZOONOTIC AND CLINICAL ASPECTS. The CNW phenotype was the Campylobacter most frequently isolated from both diarrheic and healthy dogs. So far we have not encountered such strains in man or in other animal species. The ability of the CNW strains to grow at 42°C on selective medium designed for C. coli and C. jejuni implies that they should be recognized in the clinical bacteriological laboratory provided the catalase test is performed.

Luechtefeld and Wang (17) examined 20 canine isolates of thermotolerant Campylobacter and found that all were hippurate positive, while in the present investigation only 7 strains out of 98 hydrolyzed hippurate. The fact that the two collections of canine isolates represent different geographical areas might have a bearing on the prevalence of different kind of strains.

From dogs with enteritis, CNW strains were isolated both together with canine parvovirus and without known concurring enteropathogenic agent. If this "new" Campylobacter alone or in combination with other infectious agents can cause diarrhea in dogs or human beings remains however to be investigated.

Table 1. Characteristics of thermotolerant Campylobacter strains isolated from dogs and of type strains of Campylobacter spp.^a

Tests ^b	C. fetus subsp. C. jejuni fetus		C. coli		Canine isolates		Canine isolates		C. sputorum subsp. bubulus
	CIP ^c 5396	CIP 702	CIP 7080	2 strains	7 strains	7 strains	CIP 53103		
Growth 25°C	+	-	-	-	-	-	-	+	
Growth 42°C	-	+	+	+	+	+	-	-	
Catalase	+	+	+	+	- ^w	-	-	-	
Hippurate	-	+	-	-	-	-	-	-	
H ₂ S (TSI) ^d	-	-	-	-	-	-	+	+	
TTC ^e	-	+	+	+	-	-	-	-	
Nalidixic acid	R	S	S	S	S	S	R	R	

^aSymbols: +, positive result; -, negative result; -^w, negative or weak reaction; R, resistant; S, sensitive.

^bAll strains were positive in the nitrate reduction test and in the lead acetate test for H₂S production.

^cCollection de l'Institut Pasteur, Paris.

^dProduction of H₂S in triple-sugar-iron medium.

^eTolerance to 2,3,5-triphenyl-tetrazolium-chloride.

Table 2. mol percent G + C and DNA relatedness for canine Campylobacter isolates and reference strains.

Strain	mol% G+C ^a mean and stand.dev.	DNA homology with C303 RBR ^b 60 ⁰ ΔT _{m(e)} ^c RBR 75 ⁰		
<u>Thermotolerant canine strains</u>				
CNW ^d strain C ^e 303	35.8±0.3	100	0.0	100
CNW strain C 191		96	2.6	82
CNW strain C 231	35.6±0.2	90	2.5	73
CNW strain C 192	35.2±0.3	87	2.4	78
CNW strain C 219	35.3±0.4	84	2.5	78
CNW strain C 283	35.4±0.5	80	2.8	73
Catalase positive strain C 139	33.1±0.2	40	16.7	4
Catalase positive strain C 131	32.3±0.2	38	17.3	4
<u>Reference strains</u>				
<u>C. coli</u> CIP ^f 7080	32.4±0.3	41	16.5	4
<u>C. jejuni</u> CIP 702	31.6±0.3	39		7
<u>C. fetus</u> subsp. <u>fetus</u> . CIP 5396	34.3±0.3	17		
<u>C. sputorum</u> subsp. <u>bubulus</u> CIP 53103	31.0±0.4	19		
<u>E. coli</u> B NCTC ^g 10537	52.0	4		

^aAssuming the G+C content of E. coli B to be 52 %.

^bRelative binding ratio. Mean of two determinations. The pooled standard deviation of all mean RBRs was ±2.4. The homologous DNA reassociation ranged from 71 % to 82 %. The self-binding of the labelled DNA (6-11 %) was corrected for.

^cDifference in thermal elution midpoint after incubation at 60°C. Mean of two determinations. The pooled standard deviation was ±0.91. Δ=delta

^dCatalase negative or weakly reacting.

^eStrains from National Veterinary Institute, Uppsala, Sweden.

^fCollection de l'Institut Pasteur, Paris.

^gNational Collection of Type Cultures, London.

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PHENOTYPICAL CHARACTERISTICS OF THERMOTOLERANT CAMPYLOBACTER FROM HUMAN AND ANIMAL SOURCES

Mats Walder⁺, Karin Sandstedt[‡] and Jan Ursing⁺

⁺Department of Medical Microbiology, University of Lund,
Malmö General Hospital, S-214 01 Malmö, Sweden

[‡]National Veterinary Institute, Ultuna, S-750 07 Uppsala, Sweden

SUMMARY

A total of 314 strains of thermotolerant Campylobacter were examined with regard to ability to hydrolyse hippurate and resistance to nalidixic acid (NA), 2,3,5-triphenyltetrazolium chloride (TTC) and metronidazol. Of 168 human fecal isolates, 146 were hippurate positive, three of these were NA resistant. All of 23 strains isolated from ovine abortion were hippurate positive and NA sensitive. On the other hand 72 strains isolated from pig feces all were hippurate negative and all but 5 NA sensitive. Avian fecal strains showed a less homogenous pattern. All 314 strains but 7 were TTC resistant. Nearly all hippurate negative strains were metronidazol sensitive while the sensitivity varied between hippurate positive strains. Seventeen hippurate positive strains isolated at 42°C from raw broiler grew at 37°C only upon subculture.

INTRODUCTION

The group of thermotolerant Campylobacter defined as campylobacters growing at 42°C but not at 25°C includes two recognized species, C. jejuni and C. coli (13), both specific epithets originally given to bacteria isolated from diarrheic animals (8, 4). When the genus Campylobacter was revised by Véron & Chatelain (17) the two species were redefined as the original strains were lost and existing descriptions inconclusive. They defined C. jejuni and C. coli by temperature limits of growth, sensitivity to nalidixic acid and tolerance to 2,3,5-triphenyltetrazolium chloride. The two species were differentiated largely by quantitative differences in tolerance tests.

When it was realized that thermotolerant Campylobacter was a major cause of human enteric disease (for a review see Butzler & Skirrow (3)) the need for more useful differential criteria became evident. The existence of nalidixic acid resistant thermotolerant strains observed by Skirrow & Benjamin (14) presented an additional problem. The most promising recent approach has been the use of the hippurate test for characterizing C. jejuni (6).

MATERIAL AND METHODS

BACTERIAL STRAINS. All cultures studied and their sources of origin are listed in Table 1. Criteria for inclusion in the material were Campylobacter-like bacteria growing at 42°C but not at 25°C.

The 168 human fecal strains were collected from consecutive cases of enteric disease at Malmö General Hospital. The strains from raw broiler were obtained by swabbing the interior of broilers from four different retail dealers in Malmö.

Remaining strains were isolated at the National Veterinary Institute, Ultuna. Most of the animals represented different farms. The origin of equine and bovine strains was feces from animals with enteritis. The cultures from pigs, poultry and wild birds were isolated from the intestinal content or intestinal mucosa at autopsy of animals, most of which showed signs of enteritis. Ovine strains were recovered from gastric content or liver of aborted full term fetuses.

METHODS. Isolation of the human and broiler strains followed the procedure described by Walder (18). The animal strains were isolated according to Sandstedt et al. (12). Unless specified below the methods used for characterization of the isolates were the same as previously described (12). Cultures were incubated under microaerophilic conditions for 48 h at 37°C unless otherwise indicated. Ability to grow in the presence of 2,3,5-triphenyltetrazolium chloride (TTC) was demonstrated by two procedures, growth on blood agar containing 400 µg TTC/ml and a disc method. Filter paper discs (Biodisk, Solna, Sweden) 6 mm in diameter were soaked in 1% solution (w/v) of TTC and allowed to dry; strains with an inhibition zone diameter

on thioglycollate blood agar (Difco B 432) less than 9 mm were considered resistant. Tolerance to brilliant green (Merck) was tested on blood agar containing 10 mg/l and 30 mg/l of the dye; the plates were red after 4 and 6 days. Tolerance to 8% glucose was tested in Diagnostic Thioglycollate Broth (lab M). Sensitivity to nalidixic acid and metronidazol was tested on blood agar with discs (Biodisk) containing 30 µg and 10 µg respectively. A strain was considered sensitive to nalidixic acid if the inhibition zone diameter was 10 mm or more and to metronidazol if any inhibition zone was produced.

RESULTS

All strains were microaerophilic, gram negative, motile, curved rods with positive oxidase and catalase reaction. The tests for hippurate hydrolysis and nalidixic acid resistance showed good reproducibility; the results for all strains appear in Table 1. All strains grew readily at 37°C except the 17 broiler isolates which required several subcultures before adapting to this temperature. All strains reduced nitrate.

Of the two methods for testing tolerance to TTC, the disc test gave higher number of resistant strains and also showed better reproducibility.

Of the human strains 97% were resistant in the disc test and 89% in the growth test. The sensitive strains had a zone diameter of about 20 mm or more. The resistant strains were zone-less except strain C30 which had a zone diameter of 8 mm. TTC sensitive by the disc test were 5 human strains, M 106, M 115, M 126, M 189, M 192 and 2 isolates from cows, C 273 and C 275, belonging to the same herd.

All metronidazol sensitive strains had inhibition zone diameters of about 20 mm or more. Two of the hippurate negative human strains were metronidazol resistant, M 126 and M 146. Of the hippurate positive, 36% were resistant. Of the ovine isolates 43% were resistant. Of the hippurate positive strains originating from poultry the majority was resistant (only C 34, C 126 and C 290 were sensitive). Metronidazol resistant were also the reference strains 11168 and 11352, the avian strains C 83 and C 71 and the broiler strains A 46 and A 47 (from the same processing plant).

All strains produced H_2S demonstrated by the lead acetate strip method. Only strain C 344, an equine isolate, was positive on TSI (triple sugar iron) agar. The results of tests for tolerance to brilliant green and 8% glucose will not be given due to inconsistent results.

DISCUSSION

The different tolerance tests suggested by Véron & Chatelain (17) for separating Campylobacter jejuni from C. coli have not been extensively used in recent publications; our results with the tests for tolerance to glucose and to brilliant green were inconclusive. However, the studies of Leaper & Owen (9) and Owen & Leaper (11) gave a firm indication that hippurate hydrolysis can be used as a differentiating test. It was the only phenotypical character recognized by these authors for separating C. jejuni from C. coli. For the following discussion the assumption is adopted that hippurate positive strains represent C. jejuni, hippurate negative strains C. coli. Nalidixic acid resistant strains will be dealt with separately.

Strains of C. jejuni

A vast majority of our human isolates belonged to this species, which is in accordance with the prevailing view (7, 9, 10). C. jejuni also comprised all strains from ovine abortion, which has not been reported before. As wild birds have been suggested to be involved in the transmission of the infection to ewes (16), it is interesting that our three isolates from owls were of the same species, like 30 of 32 strains from wild birds reported by Luechtefeld & Wang (10).

Of the strains isolated from poultry 64% were hippurate positive. Skirrow & Benjamin (15) though not using the hippurate test, found that strains from poultry showed a broad spectrum of phenotypical behaviour. Only C. jejuni was isolated from broilers. These strains showed an unusual feature in that they grew at 37°C only after several subcultures. The observation is of some significance as 37°C has been suggested as incubation temperature for thermo-tolerant Campylobacter (2).

The first strains described as C. jejuni (8) were isolated from bovine feces as also the type strain, CIP 702. Only 2 of 6 bovine isolates in this study were hippurate positive. A strain with identical features was isolated from feces of a diarrheic horse. Thermotolerant Campylobacter associated with equine enteric disease has been reported (1).

Skirrow & Benjamin (15) introduced the metronidazol resistance test for the characterization of Campylobacter. For C. jejuni the property seems to be variable and of possible value in epidemiological situations.

Strains of C. coli

Although the bacterium isolated by Doyle (4) was nitrate negative, the name C. coli continued to be generally used for thermotolerant porcine campylobacters in spite of their positive nitrate reaction. Seven of the nine isolates used by Véron & Chatelain (17) were from porcine feces. All our porcine strains were hippurate negative, which is in agreement with the results of Luechtefeld & Wang (10). Also Skirrow and Benjamin (15) could show that the phenotype prevalent in pigs was less often met with in other animals. In our study C. coli was also isolated from humans (22 strains), poultry (8 strains) and cattle (1 strain).

It appeared that almost all strains identified as C. coli were sensitive to metronidazol; two human strains (one of them TTC sensitive) were resistant. This is at variance with the results of Skirrow and Benjamin (15), who found most porcine strains metronidazol resistant, but who mention a possible explanation in the extensive use of nitroimidazoles in British pig rearing. This assumption is supported by the fact that the use of antibiotic drugs in Swedish pig rearing is restricted.

Strains resistant to nalidixic acid

When Skirrow and Benjamin (15) introduced the NARTC concept (nalidixic acid resistant thermophilic campylobacter) they considered this group a homogenous entity of metronidazol resistant, mainly TTC sensitive strains most of which were isolated from seagulls. Leaper & Owen (9) examined 8 of these isolates

and found them TTC resistant and hippurate negative. Hébert et al. (7) reported that also 4 nalidixic acid resistant hog isolates, were hippurate negative.

Our study included 3 hippurate positive, nalidixic acid resistant strains, all metronidazol resistant and all isolated from human feces. The isolate with reactions in closest agreement with the NARTC-phenotype was the avian strain C 71. The 9 remaining nalidixic acid resistant isolates were all metronidazol sensitive. One of them, an equine strain, differed from all other strains in the study by producing a large amount of H_2S . Thus, it had all studied properties in common with the bacterium C. faecalis isolated by Firehammer (5) from ovine feces.

General discussion

Although growth at 42°C but not at 25°C has proved to be a useful criterium for campylobacters isolated mainly from intestinal tract, these bacteria seem to have few other phenotypical traits in common. Leaper & Owen (9) found that the content of fatty acids separated thermotolerant campylobacters from C. fetus. Several authors have used the TTC tolerance test (9, 10, 15, 17) but with somewhat different results, probably a consequence of different test methods. Our data largely agree with these of Leaper & Owen (9) and Luechtefeld & Wang (10), implying that TTC resistance is a property shared by most thermotolerant campylobacters. On the other hand, Sandstedt et al. (12) recently described a group thermotolerant Campylobacter strains that were isolated from dog feces and genetically separated from other campylobacters. These strains were TTC sensitive and had a weak or negative catalase reaction.

The nalidixic acid resistant thermotolerant strains remain a somewhat enigmatic group of organisms isolated mostly from birds (15). To our knowledge no hippurate positive nalidixic acid resistant strains have previously been reported. Our 3 strains are all of human origin and it is tempting to regard those isolates simply as resistant strains of C. jejuni.

Further genetic investigations will throw light on the relationship between the different thermotolerant campylobacters and indicate where there is need for additional differential phenotypic test.

Table 1. Origin of Campylobacter strains examined

Strains	Hippurate positive		Hippurate negative	
	Nalidixic acid	Nalidixic acid	Nalidixic acid	Nalidixic acid
Source of origin sensitive resistant sensitive resistant				
Reference strains ^a				
CIP 5396 ^b	<u>C. fetus</u> subsp. <u>fetus</u>	ovine		1
CIP 702 ^b	<u>C. jejuni</u>	bovine	1	
NCTC 11168	<u>C. jejuni</u>	human	1	
CIP 7080 ^b	<u>C. coli</u>	porcine		1
NCTC 11353	<u>C. coli</u>	porcine		1
CIP 53103 ^b	<u>C. sputorum</u> subsp. <u>bubulus</u>	bovine		1
NCTC 11352	<u>Campylobacter</u> sp. (NARTC ^c)	avian (Larus argentatus)		1
Field isolates				
M3-16, M18-31, M33, M35, M37-38, M40-55, M57-83, M85, M88-94, M96, M98-99, M102, M104-113, M115-122, M124-126, M128-136, M138-148, M150-152, M154-156, M158, M160-161, M163-167, M169-175, M177-178, M180-186, M189-193, M196-200		human	143	3 ^d
C5, C7-8, C10-11, C28, C30, C33, C36, C39-45, C47-56, C58-59, C78-82, C84-92, C100, C105-110, C115, C117, C119-120, C127-128, C164-165, C172-174, C176, C184, C189, C218, C244-46, C296, C312-314, C122.		porcine		67
C344, C448		equine	1	5 ^f
continued				1 ^g

Table 1. Origin of Campylobacter strains examined

continued				1 ^h	3 ⁱ
C269, C272, C273, C275, C287, C310		bovine	2		
C66-68, C72-77, C93-96, C237-239, C252, C452-453, C461-464		ovine fetus	23		
C17-18, C34, C61, C97-99, C101, C126, C278-282, C288-291, C318-319		avian (poultry)	14	g ^k	
A528, A530, A532, A535, A536, A250-251, A253-258, A1111-1112, A46, A47		avian (raw broiler)	17		
C69, C83		avian (Strix aluco)	2		
C60		avian (Asio otus)	1		
C71		avian (Gavia stellata)			1

- a CIP: Collection de l'Institut Pasteur, Paris, NCTC: National Collection of Type Cultures, London
M: Malmö General Hospital. C: National Veterinary Institute, Uppsala. A: City Health Laboratory, Malmö
- b Type strain
- c Nalidixic acid resistant thermophilic Campylobacter
- d M26, M83, M160
- e M4-5, M7, M38, M48, M50-51, M76, M89-94, M109, M126, M142, M146, M154, M161, M192, M197
- f C30, C120, C128, C176, C314
- g C344
- h C310
- i C269, C273, C275
- k C17-18, C63-64, C97-99, C101

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BASE COMPOSITION AND SEQUENCE HOMOLGY OF DEOXYRIBONUCLEIC ACID OF THERMOTOLERANT CAMPYLOBACTER FROM HUMAN AND ANIMAL SOURCES

Jan Ursing⁺, Mats Walder⁺ and Karin Sandstedt[‡]

⁺Department of Medical Microbiology, University of Lund,
Malmö General Hospital, S-214 01 Malmö, Sweden

[‡]National Veterinary Institute, Ultuna, S-750 07 Uppsala, Sweden

ABSTRACT

A total of 31 Campylobacter strains, whereof 24 thermotolerant field isolates and 7 reference strains were studied. Mol% G + C was estimated by thermal denaturation and DNA-DNA-relatedness by the hydroxyapatite method. The strains identified as C. jejuni or C. coli and differentiated by the hippurate hydrolysis test formed separate, homogenous DNA homology groups. The two species were genetically more related to each other than to other Campylobacter species. Results suggest that the group of hippurate negative thermotolerant campylobacters resistant to nalidixic acid is genetically heterogenous.

INTRODUCTION

Before the recognition of thermotolerant Campylobacter as an important cause of diarrheal disease in humans, genus Campylobacter was mainly a concern of the veterinarian bacteriologist. Biochemically similar strains of thermotolerant Campylobacter have now been isolated from a wide range of hosts (7, 10). However, as this similarity is founded on very few phenotypical characteristics (4), the possibility remains that in fact their resemblance is deceptive. This question is not purely of taxonomical interest; it has also important epidemiological aspects.

The two recognized thermotolerant species, C. jejuni and C. coli are usually differentiated by the test for hippurate hydrolysis (2). In a DNA-hybridization study using 8 reference strains Owen and Leaper (5) showed that C. coli is genetically clearly separable from other Campylobacter species. In the present study 24 thermotolerant Campylobacter field isolates from human

and animal sources and 7 Campylobacter reference strains were examined by thermal melting point determination and DNA-hybridization.

MATERIAL AND METHODS

BACTERIAL STRAINS. All campylobacter strains and their sources of origin are listed in Table 1. Most of the field isolates were selected from a collection of thermotolerant human and animal strains, which have previously been characterized by phenotypical tests (10). Also included were four canine isolates examined by Sandstedt et al. (6); two of these (C 231 and C 303) had negative or weak catalase reaction.

METHODS. The methods for isolation of DNA, determination of mol percent G + C by thermal denaturation, ^{125}I -labeling of DNA and DNA hybridization have been described (6).

Due to sparse growth of the cultures, the available amount of DNA was small for most strains. The estimated specific activity of labeled DNA ranged from $1.2 \cdot 10^6$ - $2.7 \cdot 10^6$ cpm/ μg DNA. The range of homologous binding was usually 75 - 85 % and the self-binding of the labeled DNA was 2 - 6 % (occasionally up to 9 %). The relative binding rate (RBR) was calculated as percentage of homologous binding after correction for self-binding of the labeled DNA. The elutions on hydroxyapatite were performed in duplicate, occasionally 3 or 4 times. The pooled standard deviation of all mean RBRs was ± 2.9 %. Thus, the standard error of mean was ± 2.1 % when the elution was performed twice. The corresponding values for the differences in thermal elution midpoint ($\Delta T_{m(e)}$) were $\pm 0.8^\circ\text{C}$ and $\pm 0.6^\circ\text{C}$ respectively.

RESULTS AND DISCUSSION

BASE COMPOSITIONS. Means and standard deviations for mol% G + C of all isolates studied appear in Table 1. The values for 13 strains of Campylobacter jejuni ranged from 31.2 - 32.4 with a mean and standard deviation for the group of 31.9 ± 0.4 . Also the 11 C. coli strains made up a homogenous group with the range 32.1 - 33.1 and mean 32.6 ± 0.4 . These figures and also the G + C values for the typestrains of these species closely agree with the data of Owen & Leaper (5) and with the estimations of mol% G + C by chemical

methods published by Véron & Chatelain (9). The G + C content of the other typestrains were within the range of earlier published results (5, 9).

The % G + C for strain NCTC 11352 which is reference strain for the NARTC phenotype (nalidixic acid resistant thermophilic campylobacter) of Skirrow & Benjamin (7) was 31.4 ± 0.3 which is slightly lower than found by Owen & Leaper (5). However, two very similar strains, also nalidixic acid resistant and hippurate negative (C 120 and C 269), had values about 4 % higher.

INTRASPECIES AND INTERSPECIES RELATEDNESS OF CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI. From each species two DNAs were labeled; the type strain and one human field isolate. The hybridization results appear in Table 1. Both species showed high intraspecies relatedness with RBRs >80 % at 60⁰, 78 - 96 % at 75⁰ and $\Delta T_{m(e)}$ values of 0.3 - 2.8⁰C. The data support the validity of the hippurate reaction (2, 4, 10) for differentiating the two species but do not allow any further subdivision of the taxa with regard to source of origin. One strain of C. jejuni was nalidixic acid resistant (M 26).

A marked difference was found between the within-species relatedness and the between-species relatedness which were characterized by RBRs of 47 - 69 % at 60⁰C and 16 - 35 % at 75⁰C and $\Delta T_{m(e)}$ values of 7.1 - 10.1⁰C. Owen & Leaper (5) who used S1 nuclease in their study, reported very low relatedness between the C. coli type strain and C. jejuni. It is, however, known (1) that S1 nuclease methods give lower RBR than the hydroxyapatite method; furthermore, other differences between laboratories e.g. as to DNA fragment size might contribute.

RELATEDNESS OF CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI TO OTHER CAMPYLOBACTERS. The relatedness of C. jejuni and C. coli to other species of the genus was characterized by relative binding rates below 50 % at 60⁰C, insignificant levels at 75⁰C and thermal melting point differences above 10⁰C. The values indicate greater genetic distances than between C. jejuni and C. coli. Nevertheless, some degree of genetic relationship seems to exist between these species and the NARTC phenotype (NCTC 11352), the CNW strains (C 231 and C 303) and with C. fetus subsp. fetus and C. sputorum subsp. bubulus. Using the same hybridization technique, Sandstedt et al. (6)

found 40 % relative binding between labeled DNA from strain C 303 and the type strains for C. jejuni and C. coli.

The Campylobacter having the lowest relative binding with the labeled strains were the nalidixic acid resistant isolates C 120 and C 269; as mentioned above, these strains also had a significantly higher G + C content than NCTC 11352.

TAXONOMIC CONSIDERATIONS. The results of this study indicate that C. jejuni and C. coli (when the hippurate tests is used as differentiating criterium) make up genetically homogenous and separable bacterial groups. Moreover, they seem to be more genetically related to each other than to any other Campylobacter species studied, although these were represented by only a few strains. However, from the data concerning the nalidixic acid resistant thermotolerant strains with negative hippurate reaction can be inferred that this group probably is genetically heterogenous. In this context the need for more differential characteristics suitable for the routine diagnostic work on Campylobacter is a serious concern. For a pragmatic approach to classification the phenotypical properties determining the limits of any new species should permit a reasonable identification level (3).

Table 1. DNA relatedness and mol% G + C for thermotolerant Campylobacter and for reference strains.

Source of unlabeled DNA ^a	Source of origin ^b	% G + C mean $\pm$ stand. deviation ^c	Source of labeled DNA ^a									
			M 18		CIP 702 ^d				M 5		CIP 7080 ^d	
			60°C	75°C	60°C	75°C	60°C	75°C	60°C	75°C	60°C	75°C
			RBR ^e	$\Delta T_{m(e)}^f$	RBR	$\Delta T_{m(e)}$	RBR	$\Delta T_{m(e)}$	RBR	$\Delta T_{m(e)}$	RBR	$\Delta T_{m(e)}$
<i>C. jejuni</i>												
M 18	H	32.4 $\pm$ 0.3	100	0.0	100		97	1.9				
M 24	H	31.6 $\pm$ 0.1	100	1.4			100	1.8				
M 14	H	31.3 $\pm$ 0.3	99	1.9	94							
NCTC 11168	H	32.0 $\pm$ 0.2	96	1.8	96		89	1.8				
M 53	H	32.2 $\pm$ 0.2	89	2.7	87		96	2.2				
M 66	H	31.8 $\pm$ 0.1	87	2.6	86		91	2.8				
M 26	H	32.2 $\pm$ 0.2	86	1.8	86				67	10.1		
CIP 702 ^d	B	31.6 $\pm$ 0.3 ^g	99	1.6	95		100	0.0				
C 237	O	32.4 $\pm$ 0.3	90	2.3	88		81					
C 69	Av	32.0 $\pm$ 0.2	97	2.2	88							
C 126	Av	32.0 $\pm$ 0.2					95	1.9				
A 1112	Br	31.4 $\pm$ 0.2					93	2.2				
A 535	Br	31.2 $\pm$ 0.2	94	2.8			81	2.3				
continued												
									50	8.8		
							69	9.6	23			
							47	7.1				
							61	8.1	27			
							55	9.8	23			
							58	9.0				
							60	8.5	24			
											61	9.1
											59	8.6
												22
												16

Table 1. DNA relatedness and mol% G + C for thermotolerant Campylobacter and for reference strains.

continued														
<u>C. coli</u>														
M 5	H	32.6 ± 0.1	65	8.1	35	64	7.3	29	100	0.0	100	86	1.4	89
M 93	H	32.1 ± 0.1							94	1.7	95			
M 48	H	32.3 ± 0.2							92	1.6	88			
M 4	H	33.1 ± 0.3							84	0.3	85			
CIP 7080 ^d	P	32.4 ± 0.3 ^g	65	8.7	22	55	8.9	25	88	1.2	86	100	0.0	100
NCTC 11353	P	32.6 ± 0.2	67	8.8	29	68	8.5	24	97	0.8	90	85	1.4	86
C 58	P	32.3 ± 0.1							91	1.1	85			
C 43	P	33.0 ± 0.2							82	0.5	89			
C 131	C	32.3 ± 0.2 ^g							93	0.4	87			
C 139	C	33.1 ± 0.2 ^g	59	8.4	29				81	0.4	85			
C 99	Av	32.4 ± 0.2							91	1.9				
<u>C. species</u>														
NCTC 11352	Av	31.4 ± 0.3	40	11.9	10	29		11				26		6
C 120	P	35.8 ± 0.2				15			11			18		
C 269	B	35.0 ± 0.1	17			17			9			8		
C 231	C	35.6 ± 0.2 ^g	36	15.0	9	40	15.3		44	11.6	7	26		
C 303	C	35.8 ± 0.3 ^g				43	15.4		44	13.9	7	23		
continued														

Table 1. DNA relatedness and mol% G + C for thermotolerant Campylobacter and for reference strains.

continued				
<u>C. fetus subsp. fetus</u>				
CIP 5396 ^d	0	34.3 ± 0.3 ^g	24	28
<u>C. sputorum susp. bubulus</u>				
CIP 53103 ^d	B	31.0 ± 0.4 ^g	25	25
<u>E. coli B</u>				
NCTC 10537		52.0	5	2

^aA: City Health Laboratory, Malmö. C: National Veterinary Institute, Uppsala. CIP: Collection de l'Institut Pasteur, Paris. M: Malmö General Hospital, Malmö. NCTC: National Collection of Type Cultures, London.
^bAv: avian, B: bovine, Br: broiler, C: canine, H: human, O: ovine, P: porcine
^cAssuming % G + C of E. coli to be 52.0
^dType strain
^eRRBR: relative binding rate; homologous binding considered 100
^f $\Delta T_m(e)$: difference in thermal elution midpoint between homologous and heterologous duplexes
^gpublished earlier (5)

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Susceptibility of *Campylobacter fetus* subsp. *jejuni* to Twenty Antimicrobial Agents

MATS WALDER

Department of Clinical Bacteriology, University of Lund, The General Hospital, Malmö, Sweden

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One hundred recent clinical isolates of *Campylobacter fetus* subsp. *jejuni* were tested by an agar dilution technique for susceptibility to each of 20 antimicrobial agents. Doxycycline and gentamicin were the most active of the drugs examined, inhibiting all strains at concentrations achievable in serum. Although the median minimal inhibitory concentration of erythromycin was low, 8% of the isolates were highly resistant. All isolates of *Campylobacter fetus* subsp. *jejuni* were relatively resistant to the β -lactam antibiotics. Some strains were highly resistant to metronidazole and tinidazole.

Campylobacter fetus subsp. *intestinalis* (14) has occasionally been recovered from blood, joint fluid, cerebrospinal fluid, and placenta of patients, especially those with compromised host defences (9, 10, 17). Using a special technique, Dekeyser et al. (4) isolated a related vibrio (9) from patients with diarrhea. With the development and application of a selective culture medium (13), isolation of *C. fetus* subsp. *jejuni* from the feces of enteritis patients (14) has been a frequent occurrence in many areas of the world (5, 12). *Campylobacter* enteritis may result from animal contacts or ingestion of contaminated food and water (14). The illness is usually characterized by an acute diarrhea, with watery or even blood-stained stools, of several days duration, with or without fever (11). Apart from diarrhea, the outstanding symptom is abdominal pain, which often persists after diarrhea has ceased.

Isolation of *C. fetus* subsp. *jejuni* from stools is not in itself an indication for antibiotic treatment, unless the patient is in acute distress. However, the organisms sometimes enter the bloodstream and then must be eradicated with antimicrobial agents. At the moment, there is comparatively little published information to guide the choice of drug for therapy (2, 3). The aim of the present investigation was to determine the susceptibility of *C. fetus* subsp. *jejuni* to commonly available antibiotics.

MATERIALS AND METHODS

Bacterial strains. Ninety-eight isolates were obtained in Malmö from patients with acute diarrhea; two reference strains were kindly provided by M. B. Skirrow, Worchester, England. After identification by standard procedures (8, 9), these strains were stored in Stuart medium at -20°C until tested.

Culture media and antibiotics. The medium used was Mueller-Hinton agar (Difco) enriched with 5% human blood. Test compounds and sources were: benzylpenicillin, lot 19351, Kabi, Sweden; ampicillin, batch 184, Astra, Sweden; mecillinam, batch G A 36375, Leo, Denmark; mezlocillin, Pt 3813C, Bayer, West Germany; carbenicillin, Al 21, Astra, Sweden; cephalothin, YV 0162, Lilly; cephalixin, lot 454-226-20, Lilly; cefuroxime, 1894-A4, Glaxo; cefotaxime, Ch-B-24, Hoechst AG, West Germany; trimethoprim, A 327 119, Roche, Switzerland; sulfamethoxazole, batch 390 060, Roche, Switzerland; clindamycin, A 7800 153, Upjohn; lincomycin, R 7700028, Upjohn; erythromycin, lot 4805-CD, Abbot; tetracycline, lot 706-2364, Pfizer; doxycycline, lot GS 3065/A/3, Pfizer; gentamicin, 225 990091-00, Schering Corp.; chloramphenicol, lot 668125 A, Parke Davis; metronidazole, 104300, Leo, Sweden; and tinidazole, Pfizer. Standard solutions of these drugs were prepared (with correction for compound impurities) and subjected to twofold serial dilution prior to incorporation of the latter in Mueller-Hinton agar.

Susceptibility testing. The inoculum was derived from a 48-h culture of *C. fetus* subsp. *jejuni* grown on peptone blood agar plates and harvested in a medium containing, per liter of distilled water: Trypticase, 170 g; Phytone, 30 g; sodium chloride, 40 g; potassium nitrate, 10 g; sodium formate, 10 g; and hemin, 30 mg. The suspension was then diluted in this medium so that there were 10^6 to 10^7 organisms per ml. With a multipoint inoculator (15), 2 to 3 μl of the diluted bacterial suspension was applied to the surface of the antibiotic agar plates. These were incubated for 48 h at 37°C in gas jars containing at most 5% O_2 in addition to a gas mixture of N_2 , H_2 , and CO_2 . Minimal inhibitory concentrations (MICs) were recorded as the lowest antibiotic concentrations that gave no visible growth.

RESULTS

The range of MIC values for each of the 20 antimicrobial agents tested and the concentrations of these drugs required to inhibit 90 (MIC₉₀) and 50% of the test strains are presented

TABLE 1. Susceptibility of *C. fetus* subsp. *jejuni* to various antimicrobial drugs

Drug	Range	Inhibitory concn ($\mu\text{g}/\text{ml}$ of medium)	
		For % of strains	
		50	90
Benzylpenicillin	2.5 - 40	5	20
Ampicillin	2.5 - 40	5	20
Mecillinam	2.5 - 40	5	20
Mezlocillin	20 - 160	40	80
Carbenicillin	5 - 80	20	40
Cephalothin	>160	>160	>160
Cephalexin	40 - >160	160	>160
Cefuroxime	40 - >160	80	>160
Cefotaxime	5 - 40	10	40
Trimethoprim	>160	>160	>160
Sulfamethoxazole	5 - 80	20	40
Clindamycin	0.1 - >50	0.4	0.8
Lincomycin	1.6 - >50	6.2	12.5
Erythromycin	0.2 - >50	1.6	3.1
Tetracycline	0.4 - 6.2	0.8	3.1
Doxycycline	0.05 - 3.1	0.2	1.6
Gentamicin	0.4 - 3.1	0.8	1.6
Chloramphenicol	3.1 - 12.5	6.2	12.5
Metronidazole	0.4 - >25	3.1	>25
Tinidazole	0.4 - >25	1.6	>25

in Table 1. These data show that, as a group, the β -lactam antibiotics had low activity; MIC₉₀ concentrations of 20 $\mu\text{g}/\text{ml}$ or larger were required to inhibit growth of the test strains. Clindamycin, doxycycline, and gentamicin were clearly the most active of the 20 drugs, with erythromycin and tetracycline slightly less effective. Trimethoprim and sulfamethoxazole were inactive. Metronidazole and tinidazole had marginal activities. Only three drugs, doxycycline, tetracycline, and gentamicin, were active against all test strains at concentrations attainable in serum with standard dosage. Chloramphenicol had an MIC₉₀ of 12.5 $\mu\text{g}/\text{ml}$. This concentration is at the upper range of levels that can be attained in serum with oral dosage.

DISCUSSION

The results of this study on the antimicrobial susceptibilities of 100 strains of *C. fetus* subsp. *jejuni* are in general agreement with those obtained by Butzler et al. (2), on 7 strains of *C. fetus* and 114 of related vibrios, and by Chow et al. (3) on 11 strains of *C. fetus*. The variations in susceptibility recorded in this study were greater than were reported previously.

Erythromycin has been reported as the drug of choice for treatment of *Campylobacter* infections (1, 11, 13). The present investigation indicates a high degree of susceptibility to erythromycin for many of the clinical isolates of *Campylobacter*, with median MIC values of 1.6 $\mu\text{g}/$

ml. It is, however, of great interest that approximately 8% of the isolates were resistant to erythromycin, lincomycin, and clindamycin. Butzler et al. (2) reported one strain resistant to erythromycin in 1974. Because of this resistance, the use of these macrolide antibiotics in life-threatening infections caused by *Campylobacter* is contra-indicated. This position is further supported by the fact that the resistant strains were from four endemic outbursts without any evident connection.

C. fetus subsp. *jejuni* is fairly resistant to all β -lactam antibiotics tested. With median MICs of 5 $\mu\text{g}/\text{ml}$ or greater, penicillins are not the drugs of choice for treatment of infections caused by *Campylobacter*. The MIC₉₀ values for mezlocillin and all cephalosporins are higher than serum concentrations that can be attained with standard dosage.

The activity of metronidazole and tinidazole against *Campylobacter* is of some, although limited, interest. Until recently, these and other nitroimidazole derivatives were considered to be active only against obligate anaerobes. In this study, metronidazole and tinidazole exhibited activity at concentrations that could be attained in serum with dosages of 2 g/day (6).

ADDENDUM

After this study was completed and submitted for publication, Vanhoof et al. published a study on 95 strains of *C. fetus* subsp. *jejuni* against 29 antimicrobial agents (R. Vanhoof, M. P. Vanderlinden, R. Dierickx, S. Lauwers, E. Yourassowsky, and J. P. Butzler, Antimicrob. Agents Chemother. 14:553-556, 1978. Their results were in accordance with those reported above.

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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ANTIBODIES AGAINST *CAMPYLOBACTER JEJUNI*, AND ITS CLINICAL APPLICATION

MATS WALDER and ARNE FORSGREN

Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden

Walder, M. & Forsgren, A. Enzyme-linked immunosorbent assay (ELISA) for antibodies against *Campylobacter jejuni* and its clinical application. Acta path. microbiol. immunol. scand. Sect. B,

Antibody response to *Campylobacter jejuni/coli* (CJC) was investigated, using an enzyme-linked immunosorbent assay, ELISA. With a mixture of lipopolysaccharide from two CJC strains as antigen in ELISA, all 24 tested rabbit anti-CJC sera showed high antibody levels. However, only 70% of sera from patients with *Campylobacter* enteritis demonstrated an antibody response against the combined LPS antigen, using paired sera. In addition, the results obtained suggested non-specific binding of human immunoglobulin. When 24 formalinized whole CJC bacteria were used as antigen in ELISA, all corresponding rabbit antisera reacted with one strain (M14). Essentially no unspecific binding of human immunoglobulin was obtained. Antibodies were detected in sera from healthy blood donors and at a lower level in sera from children, suggesting early immunization. In 67 enteritis patients with positive stool cultures for CJC, a significantly increased level of IgG antibodies could be detected in single or paired serum samples from 82% of the patients. An IgG titre increase occurred early in the course of infection, suggesting a boosting of an earlier immunization. IgM antibodies could be detected in the same sera in 77% of the patients. Considering both IgG and IgM analyses of the enteritis sera, 94% of the patients were positive in *Campylobacter* ELISA serology compared with only 5% of healthy controls.

Key words: *Campylobacter jejuni*; antibodies; ELISA; clinical application.

M. Walder, Department of Medical Microbiology, University of Lund, Malmö General Hospital, S-214 01 Malmö, Sweden.

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Campylobacter jejuni and *Campylobacter coli* are now well-established causes of acute enteritis in humans (4, 18) and this has been reported from all continents. The two enteropathogenic species within *Campylobacter* will in the following together be referred to as CJC. In southern Sweden, CJC is a more common cause of bacterial diarrhoea than *Salmonella*, *Shigella* and *Y. enterocolitica* together (22).

Many serological reports of CJC have been concerned with schemes for the differentiation of strains within the species CJC to facilitate tracing of

the origin of infections (17). Detection of antibodies is also of diagnostic value but previously serological tests have only been used to a limited extent in the diagnosis of infection. Using autologous isolates as antigen in complement fixation test (3), indirect fluorescent antibody test (1) and whole cell agglutination (18), an antibody response could be detected in sera from 89–90% of patients with CJC enteritis.

More recently, *Watson & Kerr* reported that with two reference strains and a combination of agglutination assay, immunofluorescence and complement fixation, positive reactions were obtained for 90%

in subsequent tests. On the basis of these experiments a concentration of 8×10^4 CFU/ml was chosen for coating.

In LPS ELISA, LPS diluted in carbonate buffer was used for coating the plates. The optimal antigen concentration for sensitization was determined by chess board titration against pre-immune and hyper-immune sera specific for CJC, *E. coli* and *Y. enterocolitica*. The antigen dilution of 1 µg/ml LPS showed the greatest difference between positive and negative sera and was chosen for coating.

Sensitization with antigen of the polystyrene was allowed to proceed overnight at room temperature by passive absorption. The plates were then washed 3 times for 3 min in PBS Tween (Phosphate buffered saline pH 7.4, 0.05 % Tween 20 and 0.5 % BSA) and were shaken dry. A quantity of 0.3 ml of the test sera diluted in PBS Tween was added and incubated in the wells for 2 h at 37 °C. The plates were washed and shaken dry again.

Enzyme-labelled antiglobulin conjugate (0.3 ml) was then added to the plate and incubated for a further 2 h period at 37 °C. The conjugates used were commercially available alkaline phosphatase labelled rabbit anti-human immunoglobulin G (heavy chain specific) and alkaline phosphatase conjugated swine antibodies against rabbit IgG (Orion Diagnostica, Helsinki, Finland). The conjugates were diluted in PBS Tween buffer and the dilutions used were adjusted to give optimal values in this test system. Conjugates were stored in the concentrated form at +4 °C until used.

The substrate para-nitrophenyl phosphate, 0.3 ml in each well, was made up freshly before use. Sigma 104 phosphate substrate was dissolved, 1 mg/ml, in diethanolamine buffer (97 ml diethanolamine, 843 ml H₂O, 60 ml NaHCl, 100 mg MgCl₂ × 6 H₂O and 0.2 mg NaN₃). Hydrolysis of the substrate was measured in a 9 channel Titertek Multiskan plate-reader (Flow Laboratories) at 405 nm, 12.5, 25, 50, and 100 min after the reaction was started. The absorption measured was proportional to the rate of enzyme hydrolysis, which was related to the amount of antiglobulin attached to the specific antibody that had complexed with the antigen on the plate. Since the antiglobulin concentration and antigen are constant, the absorbance values can be used as a measure of the specific antibodies in test samples.

An inhibition test as a specificity control was performed with rabbit anti-CJC sera diluted 1/500 and incubated with whole cell antigen and soluble LPS antigens for 30 min at 37 °C before assay.

ELISA-titres. The test sera in triplicates were tested in phosphate buffered saline in dilutions of 10⁻²–10⁻⁶, using tenfold dilution steps. The end-point titre defined as the reciprocal dilution giving an absorbance of 0.1 at 405 nm after incubation for 100 min was registered as the end-point titre.

The absorbance values for human serum samples in triplicate at 405 nm per 100 min multiplied by the constant dilution factor (5×10^2 in the linear part of the dilution curve for human sera), were noted and referred to as relative ELISA titres (6). The mean values were

used and the accuracy of the determination was $\pm 2\%$ (s.e.m.).

In all human ELISA tests two reference sera were analysed, one from a blood donor without known disease and one from a patient with known *Campylobacter* enteritis. The absorbance of the positive reference serum was in each experiment adjusted to the same value in order to reduce between-assay variations. The values of test sera were adjusted in the same relation.

Statistical methods. The mean relative IgG ELISA titres against CJC of sera obtained from blood donors, children, vegetarians and patients were compared by analysis of variance, and deviations were expressed as $\pm$ s.e.m. (standard error of the mean) and 95 % confidence intervals were calculated. Differences in relative mean ELISA IgG titres against *Campylobacter jejuni* of paired sera obtained from patients in the acute and convalescent phase of the disease were studied by Student's two-tailed t-test.

Criteria for positive ELISA titres. Mean IgG or IgM titre above 95 % confidence interval for 100 healthy blood donors was used for one patient serum.

For paired serum samples with relative titres below 95 % confidence limit for 100 blood donors but with at least one of the paired sera with relative titre > 75 , the following criteria were set up: IgG titre differences between sera of more than $\pm 22\%$ (2 SD) and IgM titre differences of more than $\pm 28\%$ (2 SD) were considered indicative of a recent campylobacter infection. This was based on experiments using paired sera from 50 patients with respiratory tract disease.

Sensitivity, specificity and effectivity were calculated according to the rules of Galen & Gambino (8).

RESULTS

LPS ELISA

When an ELISA was carried out on 24 rabbit CJC hyper-immune sera against 10 *Campylobacter* LPS antigens, many of the sera were reacting with 5 of the LPS preparations (Table 1). The results in the table suggest that a combination of only two of the LPS antigens with ELISA would suffice to detect antibodies against *Campylobacter* in all of the 25 rabbit hyper-immune sera. On the basis of these experiments a fixed combination with equal parts of the two LPS antigens (CJC M1 and M8) was used.

The LPS preparation was tested for contents of fatty acids by gas-liquid chromatography (Docent Björn Åkesson, Department for Medical Chemistry, Lund). The analysis showed a fatty acid content of 14 % with no detectable β -hydroxy-myristic acid (β -OH-14:0) but myristic acid (14:0) 18 %, palmitic acid (16:0) 64 % and oleic acid (18:1) 13 %.

Test for specificity of the combined LPS antigen was carried out with rabbit hyper-immune sera against 24 CJC strains, *C. fetus ss fetus*, *C. fetus ss venerealis*, *E. coli*, *Y. enterocolitica* and pre-immune sera. The CJC hyper-immune sera had by far the

of patient sera and 14% of healthy controls (23). Kosunen *et al.* (13), using tube agglutination test with both boiled and formalinized antigens, reported that a combination of four reference strains gave positive titres in 91% of patients and 4% of control sera. Mosimann *et al.* (15), using a pool antigen extracted at high pH from three fresh strains, reported 90% of patient sera positive and 8% of control sera. Svedhem *et al.* (21), using a surface antigen pool from two strains in DIG ELISA, reported 90–100% of patient sera positive while healthy controls were positive in 0–35%.

The object of the present study was to find an antigen giving an immunological reaction with antisera against most isolates of CJC. The specificity was assayed with the enzyme-linked immunosorbent assay technique (ELISA). Another purpose of this investigation was to determine with ELISA the level of antibodies to the chosen antigen preparation in sera from CJC enteritis patients and different control groups. The ELISA method was chosen because it is usually 10–100 times more sensitive (5) than tube agglutination (Widal), which has conventionally been used for titration of antibodies to intestinal pathogens (25).

MATERIALS AND METHODS

Bacterial strains. 24 consecutive epidemiologically unrelated strains of CJC from patients with acute enteritis were identified, using the criteria that have been described previously (22). Eleven of the strains were negative in hippurate hydrolysis test (10), indicating that they were *Campylobacter coli*. The strains were grown micro-aerophilically on peptone blood agar plates at 37 °C. CJC strains used for preparation of combined LPS antigen named CJC M1 and CJC M8 and strain used in whole cell ELISA CJC M14 were all positive in hippurate hydrolysis test. In tests for inter-species cross-reaction *Campylobacter fetus ss venerealis* CIP (Coll. Inst. Past.) 6829 and *Campylobacter fetus ss fetus* CIP 5396 were used. *Y. enterocolitica* (My 0 Winblad 0 type 3 biotype 4) and *E. coli* 026:B6 were used in specificity tests.

Preparation of test antigens. Antigen material of CJC for whole cell ELISA was made by harvesting bacteria grown on peptone blood agar plates for 48 h in 0.9% saline containing 0.4% formaldehyde. The suspensions were left at room temperature for 18 h. The bacteria were then washed and suspended in 0.9% saline containing 0.2% formaldehyde (26). Before use, a suspension with an opacity standardized to match Brown's tube no. 2 was prepared from the stock solution.

Lipopolysaccharide antigens from CJC, *E. coli* and *Y. enterocolitica* were prepared by hot phenol water extraction (Westphal *et al.* (24)) of acetone-dried bacteria, according to Sutherland (19). The LPS was purified by

ultra-centrifugation at $100,000 \times g$ for 4 h or until no specific absorption at 260 or 280 nm could be detected, indicating that no RNA or protein content was present.

Rabbit sera. Antisera were prepared against 24 CJC strains, one *Campylobacter fetus ss venerealis* and one *Campylobacter fetus ss fetus*, by immunizing rabbits of either sex (2.5–3.5 kg) with non-living untreated bacteria. Two rabbits were immunized with each bacterial strain. The starting dose was 0.5×10^8 bacterial/ml. The dose was increased in two-fold steps every second day to a final dose of 0.8×10^9 bacteria/ml. Booster injections of 1.0×10^9 bacterial/ml were given 4 and 6 weeks after the initial dose. Rabbits were bled by venipuncture before immunization and two weeks after the last booster dose. Antisera against *E. coli* and *Y. enterocolitica* were raised in a conventional way (26).

Human sera. Sera from the following groups were assayed for antibodies to CJC: 100 consecutive registered blood donors of the blood bank, Malmö General Hospital, 29 vegetarians or lacto-vegetarians, mean duration of meat abstinence 17.7 years (kindly provided by Professor Per-Arne Öckerman, Lund), 49 children with upper respiratory disease (age 0.5–12 years), 63 patient sera that were found to have high titres in other serological tests (direct agglutination of *Salmonella* and *Y. enterocolitica*, antistreptolysin, antistaphylosin, *Listeria* complement fixation and C-reactive protein), 67 patients with positive stool cultures for CJC from whom paired sera were obtained, (CJC sera were divided into five groups according to time-lapse after onset of infection, 0–10 days, 11 days–3 weeks, 4 weeks–10 weeks, 11 weeks–5 months and > 5 months), 12 patients with IgG myeloma (kindly provided by Dr. Anders Grubb, Department of Clinical Chemistry Malmö) with very low (1–3 g/l) polyclonal IgG concentration, and one patient with agammaglobulinemia (JB). A human gammaglobulin fraction 16.5% (Kabi) diluted to 25 µg/ml was also tested against LPS antigen in ELISA.

All the sera were stored in small aliquots at –20 °C and were not thawed or re-frozen more than once. Test samples were stored at –20 °C for up to one year. Repeated testing showed that no antibody activity was lost under these conditions.

ELISA procedure. Whole cell ELISA was performed with a microtitre modification (27) of the enzyme-linked immunosorbent assay (7, 9). The bacteria were formalinized as described above and the antigen was diluted in carbonate buffer, pH 9.6, 0.05 mol/l, and 0.3 ml of the solution was added to each well of disposable flat-bottom polystyrene microtitre plates (Cooke M 129, Dynatech Laboratories), which were used as solid-phase for the absorption of the antigen. The plates were incubated with suspensions of CJC, *E. coli* and *Y. enterocolitica* in different concentrations in checkerboard titrations against pre-immune and specific hyper-immune rabbit sera. The amount of antibody bound, as reflected in the shape of the titration curve, varied with the concentration of bacteria used. The dilution that showed the greatest difference between positive and negative serum was used

TABLE 1. *LPS ELISA. Titration of 24 Rabbit Immune Sera against 5 CJC LPS Antigens. Relative Titres of Dilutions*

Antiserum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	18	19	20	21	22	23	24	25
Antigen 1	+++	++	+	+++	+++	+	+++	+++	++	++	++	+++	++	-	-	++	+++	++	-	++	-	-	+	++
4	+	+++	-	+++	+++	-	+++	+++	+++	++	-	+	-	-	-	+	+	-	-	-	+	+	-	-
5	+	++	+	+++	+++	+	+++	+++	+++	+++	-	+	+	-	-	+	-	-	-	-	+	+	+	-
7	+	-	-	+++	+++	+++	+++	-	++	+++	-	+	-	-	-	-	-	-	-	-	-	+	+++	-
8	++	++	+	++	+++	+	+++	+++	+++	++	+	+	+++	+	++	+++	+	+	+++	+	+++	+++	+	-

Code: +++ Corresponding to strongest reactions against homologous antigens

++ 50-75 % of strongest reactions

+ 25-50 % of strongest reactions

- <25 % of strongest reactions

highest end-point titre (3.5×10^5 – 9×10^6) compared with all the other sera (2×10^3 – 1×10^4).

The capacity of LPS antigens to inhibit the reaction between antibodies to CJC and CJC LPS antigen was tested with ELISA. The homologous reaction was inhibited only by CJC LPS. A complete inhibition was recorded in concentrations above $1 \mu\text{g/ml}$. No inhibition could be detected with LPS from *E. coli* and *Y. enterocolitica*.

When analysing human sera, using LPS antigen in IgG ELISA, high titres were found for controls as well as patients, as seen in Table 2. The IgG mean titre was significantly higher ($p = 0.005$) in the convalescent sera (271 ± 22) compared with mean titre in acute sera (238 ± 21) from the same patients. Using CJC LPS antigen in IgG ELISA with patient sera obtained 0 to 10 days and 3 weeks to 11 weeks after onset of infection, we found that 70% of paired sera from 23 enteritis patients had a significant increase of titre between acute and convalescent phase sera. When measuring IgM titres with LPS antigen in ELISA, no significant differences between patient sera were obtained.

Sera from 12 patients with IgG myeloma, one with agammaglobulinaemia and a commercial gammaglobulin preparation ($25 \mu\text{g/ml}$) were also tested against LPS antigen in IgG ELISA. The myeloma sera and the gammaglobulin fraction gave high relative titres in ELISA, mean 225 and 180 respectively, whereas the agammaglobulinaemic serum was almost equal to buffer.

Whole Cell ELISA

In ELISA a checkerboard titration was performed with 24 CJC hyper-immune rabbit sera against the 24 corresponding formaldehyde-treated bacterial strains. High titres were found for the tested

TABLE 2. Mean Relative IgG ELISA Titres of Sera from Healthy Controls (Registered Blood Donors), And of Sera in Pairs from Patients in Acute and Convalescent Phase of CJC Enteritis against $1 \mu\text{g/ml}$ CJC LPS (Combined Antigen)

Subjects	Sera no. tested	Mean titre	($\pm$ S.E.M.)
Healthy controls	100	282	(± 13)
Patients acute phase 0–2 weeks	23	238	(± 21)
Convalescent phase 3 weeks–4 months	23	271	(± 22)

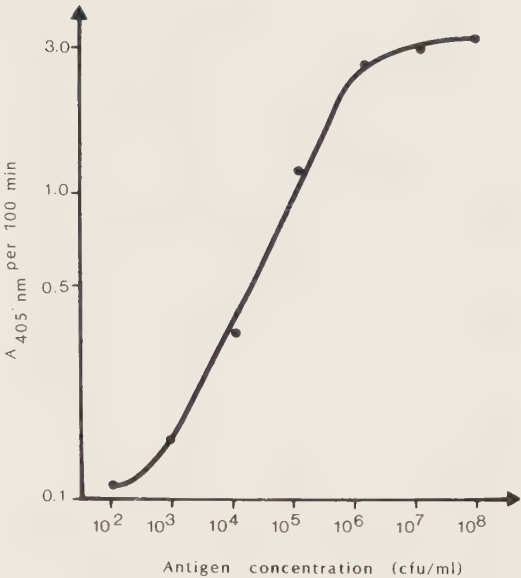


Fig. 1. ELISA whole cell antigen titration curve against typical rabbit anti CJC (C) serum 1/500.

hyper-immune sera against their homologous strains. One strain (CJC M14) also showed high positive titres against all 23 heterologous strains. Fig. 1 shows that there was a good coating ability

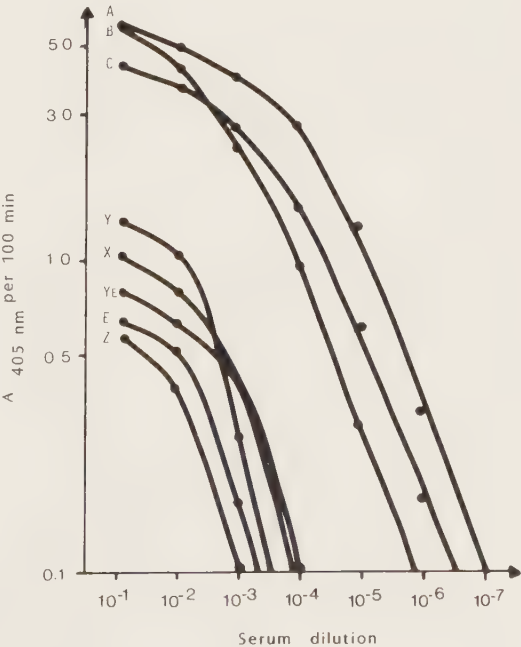


Fig. 2. Typical ELISA end point titration curves of rabbit anti CJC sera (A, B and C), anti *C. fetus ss fetus* (X), anti *C. fetus ss venerealis* (Y), anti *E. coli* (E), anti *Y. enterocolitica* (Ye) and pre-immune sera (Z) against CJC whole cell antigen.

for CJC whole cell antigen (CJC M14) in concentrations between 1×10^3 – 3×10^6 bacteria per ml. The concentration of 8×10^4 bacteria per ml was chosen in all of the subsequent tests. No sensitization was recorded in tests with pre-immune sera.

Fig. 2 demonstrates typical titration curves when rabbit hyper-immune sera against CJC (3 sera representative of 24 different hyper-immune sera), *C. fetus ss fetus*, *C. fetus ss venerealis*, *E. coli*, *Y. enterocolitica* and pre-immune sera were titrated against the whole cell antigen previously described. The CJC hyper-immune sera had by far the highest titre. Using the definition of end-point titre CJC hyper-immune sera had a more than 100-fold higher titre (8×10^5 – 10^7) than all the other sera (1×10^3 – 1×10^4).

The capacity of 3 whole cell antigens from *Campylobacter*, *E. coli* and *Y. enterocolitica* to inhibit the reaction between antibodies to CJC and CJC whole cell antigen was tested with ELISA. The homologous reaction was inhibited by CJC whole cell antigen to 90–100%. No inhibition ($< 10\%$) could be detected with whole cell antigen from *E. coli* and *Y. enterocolitica*.

There was a good correlation ($r = 0.93$) between end-point titres and relative ELISA titres both in rabbit and patient sera tests, and in the following only relative titres are used.

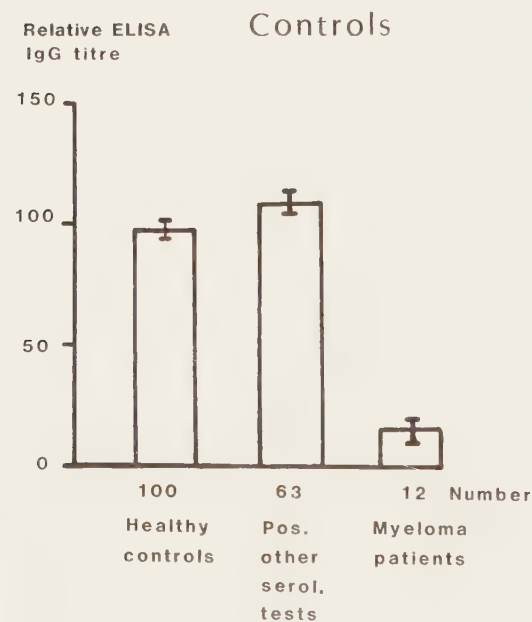


Fig. 3. Relative ELISA IgG titres of sera from healthy controls, sera positive in other serological tests and patients with myeloma against CJC whole cell antigen. Mean and SEM are denoted.

Children

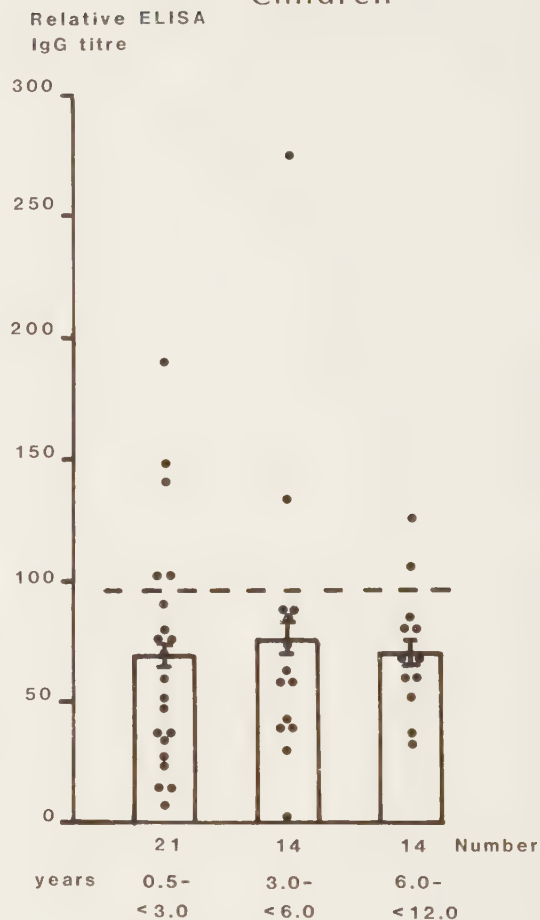


Fig. 4. Relative ELISA IgG titres of sera from children against CJC whole cell antigen. Dashed line indicate mean adult level. Individual results, mean and SEM are denoted.

Titration of Sera from Human Control Groups and from Patients with Enteritis in Whole Cell ELISA

Sera from 100 healthy blood donors were assayed for antibodies in ELISA with the use of whole cell CJC antigen.

The IgG mean titre was 96 ± 6 (s.e.m.) (Fig. 3). Patient sera with high titres in other serological tests (Fig. 3) and vegetarians (Fig. 6) showed no difference in IgG mean titre 107 ± 8 and 114 ± 11 , respectively, compared with healthy controls. Sera from 12 patients with IgG myeloma and one with agammaglobulinaemia were also tested against whole cell antigen in ELISA. The myeloma sera gave very low mean titres (15 ± 6) (Fig. 3), indicating a low degree or no non-specific reaction in the ELISA test. The agammaglobulinaemic serum was negative, i.e. equal to buffer and the

Patients with campylobacter enteritis

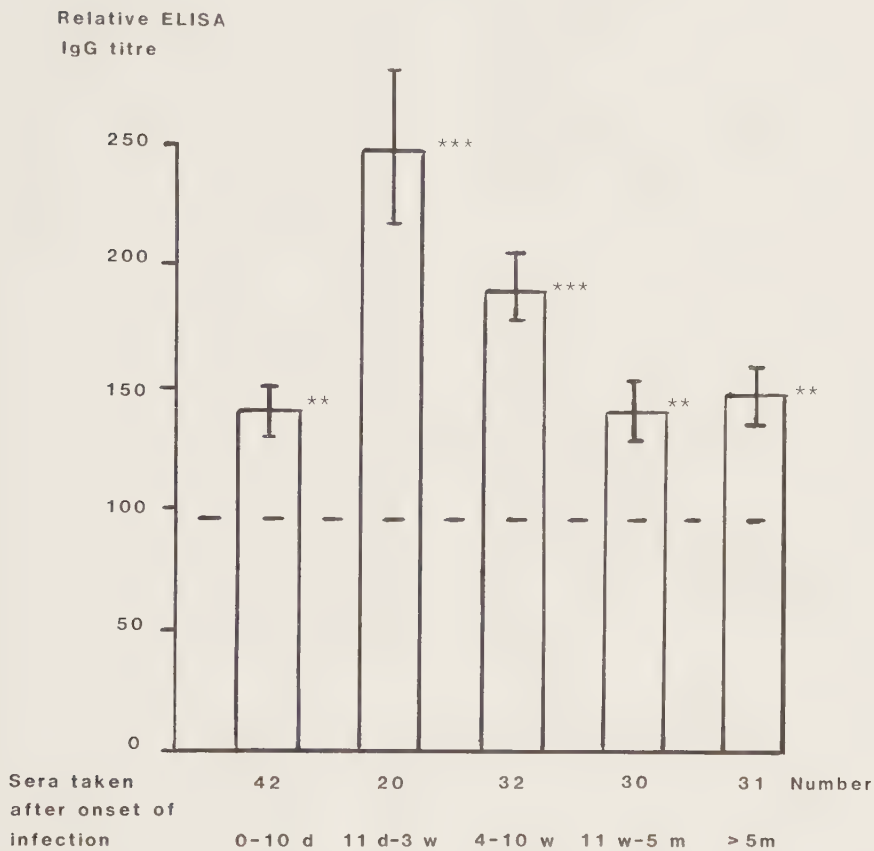


Fig. 5. Relative ELISA IgG titres of sera from patients with *Campylobacter* enteritis against CJC whole cell antigen. Dashed line indicate mean adult level. Mean and SEM are denoted and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

commercial immunoglobulin preparation equal to serum from healthy controls.

Mean titres of sera from 49 healthy children (age $\frac{1}{2}$ -12 years) were calculated and the children had significantly ($p < 0.05$) lower mean titres (68-75) compared with healthy controls. (Fig. 4). The lowest mean titre (68) was recorded in age group 0.5-3 years, the mean titre in this group being increased by the serum from three children with high IgG titres.

From 67 enteritis patients with known *Campylobacter* enteritis paired or more blood samples were obtained. The sera were placed in 5 groups according to time after onset of the infection (Fig. 5). The mean IgG titre (140 ± 15) of 42 sera collected 0-10 days after the patient fell ill was significantly ($p < 0.01$) higher than the mean titre found for healthy controls (96 ± 6) (Fig. 4). Even greater differences ($p < 0.001$) compared with

healthy controls were found for the sera collected 11 days to 3 weeks and 4 weeks to 10 weeks after onset of infection, with mean titres 248 ± 36 and 189 ± 16 respectively. For sera collected 11 weeks to 5 months and more than 5 months after the patient fell ill, mean titres were 140 ± 15 and 149 ± 15 respectively, with $p < 0.01$ for both groups compared with healthy controls.

Mean IgG titre and 95% confidence interval for 100 healthy controls was 96 (0-191). Thus single patient sera with relative titres > 191 were considered positive in the *Campylobacter* IgG whole cell ELISA test. Paired patient sera were positive in *Campylobacter* IgG serology when the titre difference was $> 22\%$, provided that at least one of the paired sera had a relative titre > 75 . With these criteria 49% of enteritis patients with CJC in stools were positive in a single serum sample and additionally 33% were positive in paired serum

Persistence of antibodies against campylobacter

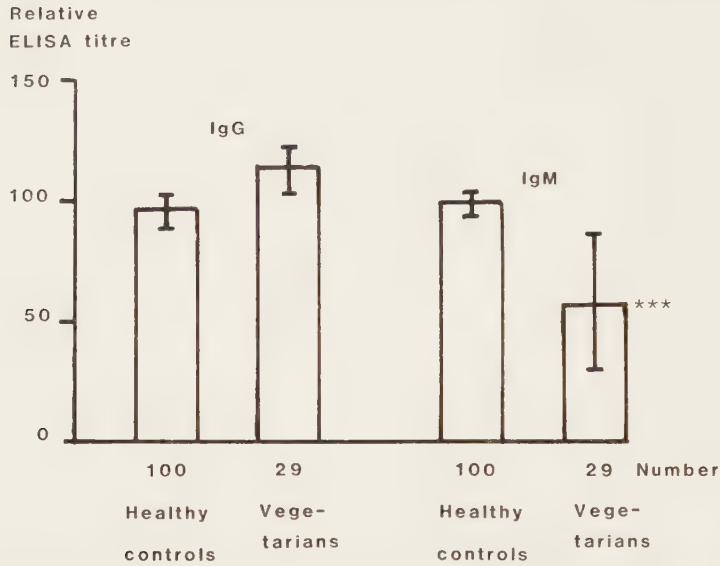


Fig. 6. Persistence of antibodies in sera from healthy controls and vegetarians in CJC whole cell ELISA. Mean and SEM are denoted and *** $p < 0.001$.

Patients with campylobacter enteritis

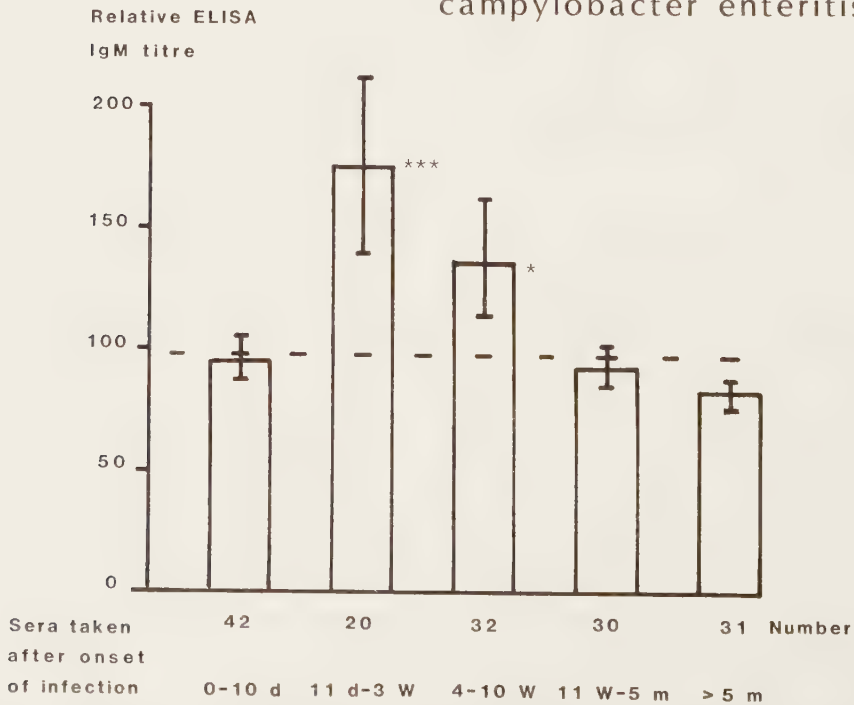


Fig. 7. Relative ELISA IgM titres of sera from patients with *Campylobacter* enteritis against CJC whole cell antigen. Dashed line indicate mean adult level. Mean and SEM are denoted and * $p < 0.05$, *** $p < 0.001$.

samples and together 82% of these patients had positive *Campylobacter* IgG whole cell ELISA serology.

Mean IgM titre in whole cell ELISA for 100 healthy controls was 98 ± 6 . Children aged 0.5–3 years had significantly ($p < 0.05$) lower mean titre (75 ± 9) compared with healthy controls, with high titres for the same 3 children mentioned above. Patient sera positive in other serological tests showed no difference in IgM mean titre (105 ± 9) compared with healthy controls. However, vegetarians had significantly ($p < 0.001$) lower mean titre (56 ± 32) compared with the same blood donors (Fig. 6).

Patients with sera collected 11 days to 3 weeks and 4 weeks to 10 weeks after onset of disease had a significantly higher mean IgM titre $p < 0.001$ and $p < 0.05$ respectively, compared with healthy controls (Fig. 7).

Mean relative IgM titre and 95% confidence interval for 100 healthy controls was 98 (0–201), thus single patient sera with relative IgM titre > 201 were considered positive in *Campylobacter* IgM whole cell ELISA serology. Paired patient sera were considered positive when the difference between pairs was $> 28\%$, provided that at least one of the paired sera had a relative titre > 75 .

Using the criteria defined above, 18% of enteritis patients with CJC in stools were positive in single sera and 76% in serology of paired sera analysed with CJC IgM whole cell ELISA. Together, 77% of these enteritis patients had positive *Campylobacter* IgM ELISA serology.

The sensitivity of the CJC whole cell ELISA serology (IgG + IgM) was calculated to be 94%, the specificity 95% and the effectivity 95%.

DISCUSSION

The development of an assay specific for antibodies against *Campylobacter coli/jejuni* is motivated by the occasional occurrence of extra-intestinal »reactive« complications, such as arthritis and glomerulonephritis, in patients with campylobacteriosis (6, 13, 14). It is generally assumed that immunological mechanisms combined with host factors are important for these complications. Antibody titrations are also very useful in epidemiological studies.

Antibody determinations in the diagnosis of bacterial enteritis are conventionally made with the antigen preparations containing the HO-antigen (25). Whole cell agglutination experiments initially showed that no useful combination of the 24 HO-antigens tested in chessboard titration against

corresponding rabbit immune sera could provide an antigen mixture cross-reacting with all serological types within CJC.

Although antibody affinity affects the results of ELISA (2), the method can be regarded as quantitative. Being sensitive, specific, and easy to perform, ELISA offers many advantages over other serological methods. An ELISA-method was therefore developed for the quantitation of IgG and IgM antibodies against CJC in human and rabbit sera.

Bacterial LPS is a potent mediator of various bacteriological effects and has antigenic properties (16). LPS can be extracted by the hot phenol water method. It has an excellent affinity for polystyrene plastic (5, 11) and was therefore tested in ELISA.

The specificity of the antigen used is of crucial importance in order to take full advantage of the increased sensitivity offered by ELISA. After having assayed 10 LPS antigens against 24 serologically different *Campylobacter* antisera in the ELISA system, 2 LPS antigens were selected owing to their broad reaction with many antisera. We demonstrated high specificity with rabbit hyper-immune sera, but with human sera we found, however, that LPS antigen in ELISA gave a high background, probably owing to nonspecific binding of gammaglobulin. Since it is reasonable to assume that all myeloma sera tested did not have antibody activities directed against *Campylobacter*, the high titres found in these sera seem to indicate that the reaction of LPS with IgG immunoglobulins is not a true antigen-antibody reaction. We could not use a single patient serum to estimate the antibody concentration in LPS ELISA because of the high background.

Therefore we tested whole cell antigen in ELISA and found one single formalinized whole cell antigen that reacted with all 24 CJC hyper-immune antisera. The antigen showed high specificity, demonstrated low background, and no cross-reactivity could be seen with hyper-immune sera against *C. fetus ss fetus* and *C. fetus ss venerealis* (Fig. 2). Rabbit *E. coli* and *Y. enterocolitica* hyper-immune sera failed to react with the CJC antigen in ELISA, and rabbit pre-immune sera also showed low activity. These results lend further support to the concept of the CJC specificity of the antigen used.

The inter-strain cross-reactivity for CJC demonstrated in Fig. 2 enables sero-epidemiological studies to be made, but also facilitates detection of the cause of enteritis in surveys of patients (12, 15, 23).

The cross-reactions and specificity of the whole cell antigen were also studied with the technique of ELISA inhibition. No substantial inhibition was seen in any heterologous system. By contrast, the

homologous *Campylobacter* reaction was inhibited to almost 100% with homologous CJC whole cell antigen. In myeloma sera very low titres were found, further indicating the specificity of the CJC ELISA.

To express the antibody content determined by ELISA as a traditional end-point titre, it is necessary to analyse serial dilutions of the serum sample. This is not only laborious, but also consumes large amounts of reagents. We, as well as others, have found a significant correlation between end-point titre and the serum dilution titre, expressed as relative titre (11). Consequently the relative ELISA titre can be considered as an equally true estimation of the antibody content as the end-point titre.

The use of whole bacterial antigen in ELISA gave several advantages over LPS antigen. Preparation of LPS is laborious and time-consuming, but technically there are essentially no differences in ELISA procedure using the two different antigens. By using the whole bacterial antigen, we detected antibodies in patient sera that we had not been able to distinguish when we used the LPS antigen.

Analysing sera from 67 CJC culture positive enteritis patients with *Campylobacter* whole cell IgG ELISA, we detected elevated antibodies in 49% of single patient sera and in 63% of paired sera and together in 82% of patients tested. With addition of IgM analyses of sera from the same 67 patients, 77% of them were positive in IgM tests, and together 94% of the 67 patients were positive in *Campylobacter* whole cell ELISA serology, when considering both IgG and IgM analyser, compared with only 5% of healthy controls.

In enteritis patients, elevated IgG and IgM titres could be detected during the first 10 days, with peaks between 11 days to 3 weeks after onset of infection. This observation that IgG antibody formation occurs early in the course of infection has also been indicated by others (21, 23).

In sera from 21 children aged between 0.5–3 years, low titres were detected both in IgG and IgM, except in 3 of them. These 3 children showed evidence of recent infection, thus increasing the mean titre. In adults we normally found antibodies: thus they seem to be immunized and boosted by *Campylobacter* antigens commonly found in meat (20).

Owing to this, CJC ELISA titres in epidemiological surveys may be difficult to interpret if there is no correlation to a recent enteritis outbreak within the last months.

The significantly lower IgM titre but normal IgG titre found in sera from the vegetarians compared with sera from healthy controls may be due to the

lack of boosting with sub-infectious doses of *Campylobacter* bacteria that ordinary meat-eaters are exposed to (Fig. 7). The IgG antibodies thus seem to persist for a long time, whereas the IgM antibodies gradually decrease with time.

On the basis of these experiments, sera from patients with enteritis of unknown etiology or with extra-intestinal »reactive« complications after enteritis can now be assayed for IgG and IgM antibodies against CJC, irrespectively of the serotype of CJC causing the illness.

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Epidemiology of Campylobacter Enteritis

MATS WALDER

From the Departments of Clinical Bacteriology and Infectious Diseases, University of Lund, Malmö General Hospital, Malmö, Sweden

ABSTRACT. *Campylobacter jejuni/coli* (CJC) was isolated from 386 patients (6.9%) of 5571 with a history of acute diarrhoea between December 1977 and June 1980. In the same study population *Salmonella* was found in 4.1%, *Shigella* in 1.7% and *Yersinia enterocolitica* in 2.1%. Only 5 (0.25%) of 2000 health controls had CJC in their stools. 53% of the patients had acquired their infection in Sweden. The peak incidence for CJC was from July to September. About 50% of the patients were between 16–35 years. Within 1 month of the acute enteritis 80% had negative stool cultures for CJC. In general, campylobacter enteritis is not a severe disease and only 11% were admitted to hospital. The most common signs were high fever in 75%, frequent watery diarrhoea in 45%, colics or abdominal pains in 84%, and fresh blood in stools in 12%. Antibiotic treatment was given in 13% and was erythromycin in 56% and doxycycline in 26% of these patients. If chemotherapy was given and the strain was sensitive, no relapse occurred within 2 weeks of the treatment. The antibiograms for 435 strains showed that the aminoglycosides, erythromycin, doxycycline, chloramphenicol and nalidixic acid were the most effective drugs. This study implies that CJC is a common cause of bacterial diarrhoea also in patients with domestic enteritis.

INTRODUCTION

Campylobacter jejuni/coli (CJC), or *Campylobacter fetus* subspecies *jejuni* (16) has been suspected of being a human intestinal pathogen for some time (9). In 1972 Dekeyser et al. (5) isolated CJC from the stools of 2 patients and the next year the same team (3) demonstrated CJC as the cause of diarrhoea in about 5% of children with enteritis. Skirrow using a selective culture medium in 1977 isolated CJC in 7.1% of stools from both children and adults suffering from acute diarrhoea (15). CJC has now been isolated from faeces of enteritis patients in many areas of the world.

The present epidemiological investigation was undertaken to describe the relative frequency, geographical distribution, seasonal variation, age and sex distribution, duration of stool carriage, symptoms and signs of campylobacter enteritis and the antibiotic susceptibility of CJC strains isolated in Malmö over 2½ years. Malmö is an urban district with 235 000 inhabitants in the south of Sweden, latitude 55° 35'.

MATERIALS AND METHODS

Isolation and identification of strains

All stool samples and rectal swabs received at the Bacteriological Laboratory, Malmö General Hospital are

routinely cultured for *Salmonella*, *Shigella* and *Yersinia enterocolitica*. Samples are cultured directly on XLD agar (20), after enrichment in Rappaport's broth (13) on LSU agar (8), and after enrichment in broth with one disc containing 30 µg cephalothin and one disc with 100 µg carbenicillin on LSU agar. Since December 1977 all stool samples are also cultured on Skirrow's medium for CJC at 42°C for 48 h in gas jars containing about 4–8% O₂ (15).

The criteria used for the diagnosis of CJC was a positive test for oxidase, catalase, and motility, plus characteristic gram stain and inhibited growth at 42°C in air and at 25°C in 4–8% O₂.

Sensitivity testing

Antibiotic sensitivity testing was performed for 435 consecutive isolates of CJC on human blood agar in gas jars using antibiotic containing discs as described by Ericsson and Sherris (6). Break points for susceptibility were those of the Swedish Reference Group for antibiotics (19).

Patients

Between December 1977 and June 1980 a total of 33 000 stool samples from 18 000 patients were investigated at our laboratory. Approximately 11 000 of the samples came from routine health controls done before employees are allowed to start working in the food industry; this group is not included in the main material.

The remaining 22 000 stool samples from about 8 000 patients were used for studying the frequency of 4 bacterial intestinal pathogens. All patients with positive cultures for CJC were analysed in studies on seasonal variation, age and sex distribution, duration of stool carriage and antibiotic susceptibility.

Symptomatology and epidemiological data from 204 patients (in all 243) with known campylobacter enteritis visit-

Table I. Distribution of bacterial intestinal pathogens: *Salmonella*, *Shigella*, *Yersinia enterocolitica* and *Campylobacter* (CJC) in stool samples investigated at the bacteriological laboratory in Malmö from December 1977 to June 1980

Pathogens	No. of isolates	No. of isolates from 5 571 enteritis patients	Isolation frequency in enteritis patients (%)			
			1978	1979	1980	31 months
<i>Salmonella</i>	395	229	4.9	3.2	3.2	4.1
<i>Shigella</i>	130	97	1.9	1.2	1.7	1.7
<i>Yersinia enterocolitica</i>	158	120	2.3	2.3	1.8	2.1
<i>Campylobacter</i>	435	386	6.2	7.8	6.8	6.9
Total	1 118	832				14.8

ing the Department of Infectious Diseases, Malmö General Hospital, were gathered from available patient records.

In a separate study during the second half of 1980 stool samples from 2 000 health controls were cultured for CJC.

RESULTS

A history of acute diarrhoeas was found for 5 571 of the about 8 000 patients included in the study, for the rest no data were available. During the same time CJC was isolated from 435 patients, 386 of whom had a history of acute enteritis; for the remaining no data were available. The incidence of campylobacter enteritis was thus 6.9%. The relative frequency of CJC compared to the frequency of other bacterial pathogens isolated during the same period is shown in Table I. There was no significant difference between years. In the 435 patients with CJC other bacterial intestinal pathogens were found in 19: salmonella in 9, shigella in 8, both salmonella and shigella in 2 patients.

As a total, 14.8% or 1 patient in 6 with acute diarrhoea could be bacteriologically diagnosed. The most common bacterial etiology of enteritis in our area was CJC which caused almost 50% of the cases. Only 5 (0.25%) persons of the about 2 000 health controls had CJC in their stools.

Geographical distribution

The records of 204 patients were examined in an attempt to locate an area of infection with CJC. 53% of the patients included in this study over 2.5 years had acquired their infection in Sweden. Most of these patients lived in the urban area of Malmö in the south of Sweden, and no special occupation proved more prevalent than another.

A recent journey (within 2 weeks) outside Scandinavia was recorded for 47% of the patients. However, 56% of the patients between 21 and 35 years had acquired their infection outside Sweden. Patients who had contracted their enteritis abroad, often mentioned countries or cities identical to popular resorts for Swedes as possible area of infection. Table II which shows the foreign areas from which the patients contracted their infection, indicates a clear predominance for the Mediterranean area.

Seasonal variation

About 65% of the infections with CJC were seen in the warmer months of the year, May–October (Fig.

Table II. Areas outside Sweden where 96 patients most probably contracted campylobacter enteritis

	%	Percentage of passengers to Swedish charter flight destinations 1978 ^a
Europe except Mediterranean countries	21	16
European Mediterranean countries	24	50
African Mediterranean countries	29	3
Africa south of Sahara	2	—
The Middle East	4	1
Canarie Islands	2	16
Asia	14	1
North America	1	0.5
The West Indies and South America	2	—

^a Regular flights not included.

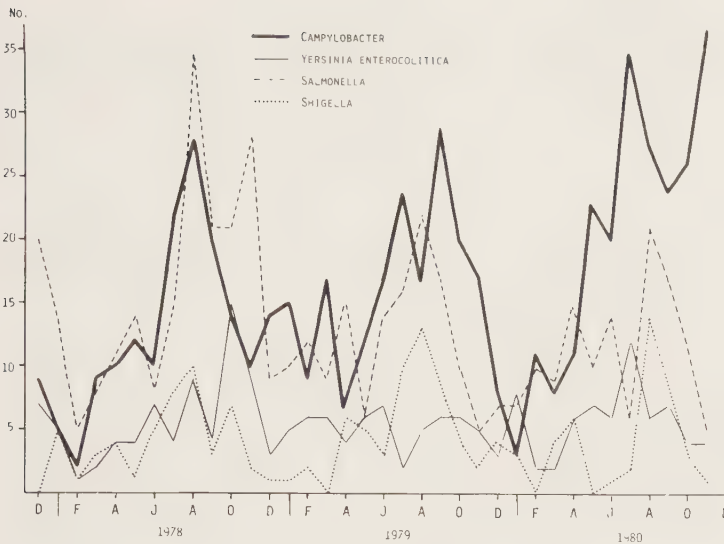


Fig. 1. No. of patients in Malmö with bacterial intestinal pathogens during 36 months.

1 shows the seasonal variation for the 4 bacterial intestinal pathogens during the time of the study plus 5 months or 3 year cycles). This seasonal variation was seen for other bacterial intestinal pathogens as well. The peak incidence for CJC was from July to September (except for 2 local epidemics in November 1980, with 11+5 patients, respectively, both outbreaks caused by improperly boiled poultry). Monthly, as a whole, there was an equal distribution between domestic and imported cases, as seen in Fig. 2. No obvious difference in seasonal

variation could be seen between the 3 years 1978, 1979, and 1980.

Age and sex distribution

More than 50% of the patients were between 16 and 35 years, mean age 27.5 years, as seen in Fig. 3. In the group of 23 hospitalized patients 45% were over 60 years old. In the material of health controls the mean age was 47 years for the 5 people found to have CJC in their stools.

Children below 10 years of age represented as

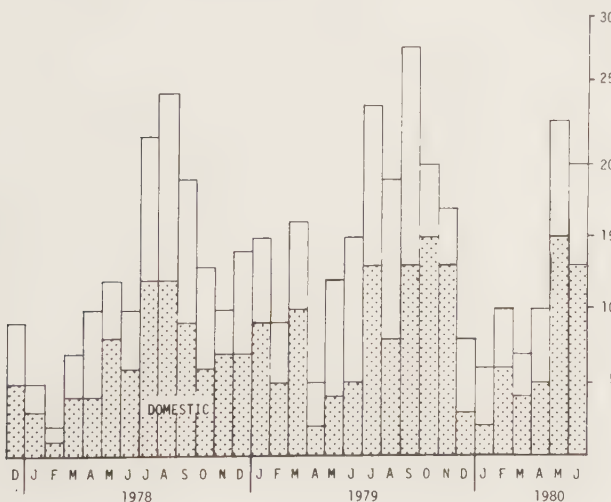


Fig. 2. Number of campylobacter cases contracted in Sweden and abroad over 31 months.

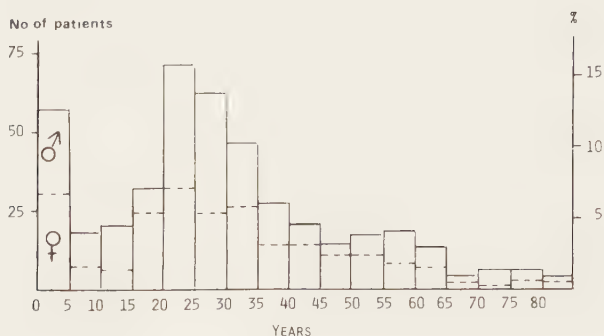


Fig. 3. Age and sex distribution for 435 patients with campylobacter enteritis.

much as 17% of all cases (Fig. 3). Four per cent of all patients were below 1 year. 8/57 children below 5 were adopted from abroad. Most of them came from South East Asia or South America. Patients over 40 years of age constituted 25% of the cases and there was an obvious decline of incidence towards old age (Fig. 3). The age distribution was similar in the 3 years analysed. A slight predominance of infections in boys under the age of 15 was seen, as in many other gastrointestinal infections, and women outnumbered men in those over 40. As a whole campylobacter enteritis was more or less equally common in both sexes.

Duration of stool carriage

For 435 patients the duration of positive stool carriage was followed by culture of stools once a week until 5 or 3 negative cultures were obtained. The stools of patients who had not been given

chemotherapy usually remained positive for about 2 weeks, mean 13.2 days, after an acute campylobacter enteritis (Fig. 4). More than 50% of the patients became permanently negative in stools after 2 weeks and over 80% were negative within 1 month. Relapses occurred in 12% after 1 negative stool culture, in 5% after 2, in 2% after 3, and in 1% after 4 negative cultures.

Only 0.7% (3 patients) excreted the organism after 3 months, and one patient 69 years old had positive stool cultures up to 128 days after the onset of enteritis. No transmission man to man could be observed.

Symptoms and signs

Hospital records of 204 adults were examined retrospectively for symptomatology. 88% of the patients with stool cultures positive for CJC had diarrhoea that was most frequently liquid or watery (Table III). Vomiting was absent or scarce in 91% of the cases.

Fever was mostly high, over 39°C in 43% of the patients. Malaise, dizziness, and nausea were almost always present. 114 patients (84%) complained of colics or other abdominal pains even before the onset of diarrhoea (Table III). Only a few, 12%, had noticed fresh blood in the stools. One patient in 10 was admitted to hospital either because of severe illness (70%), or because age and disease debilitated them (30%). The inpatients (45% > 60 years) had a registered mean nursing time of 4 days.

Four patients with reactive non-septic arthritis were seen. One was assayed and found to possess histocompatibility antigen HLA-B27 (1, 10) in HLA typing performed at the blood bank of the University Hospital in Lund.

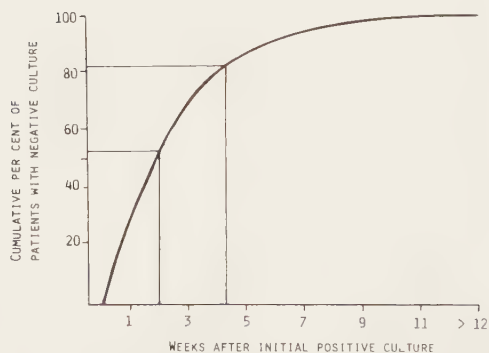


Fig. 4. Disappearance of *Campylobacter* from stools of 435 patients. Cumulative % of patients with negative culture after verified campylobacter infection.

Table III. Symptoms and signs in 204 adults with campylobacter enteritis

	%	No.
<i>Diarrhoea</i>		
> 10/day	45	77
3-10/day	43	73
1-2/day	12	21
Data missing		33
Total		204
<i>Vomiting</i>		
>2/day	9	11
1-2/day	24	31
No vomiting	67	85
Data missing		77
Total		204
<i>Fever (°C)</i>		
>39	43	71
38-39	32	52
<38	25	42
Data missing		39
Total		204
<i>Pains</i>		
Colics	60	82
Abdominal pains	24	32
No pains	16	22
Data missing		68
Total		204

Antibiotic susceptibility testing and treatment

Sensitivity tests for antimicrobial agents were performed on human blood agar by disc diffusion technique for 435 consecutive isolates of CJC from patients with positive stool samples.

All strains were sensitive to the aminoglycosides (Table IV). Resistance to erythromycin has been reported earlier (20, 21, 22), but in this extended material these strains constitute only 2.6%. They were generally resistant to clindamycin as well and on the whole approximately the same percentage of resistant strains was found for clindamycin (2.8%). Resistance to doxycycline and chloramphenicol was 2% in each case. For metronidazole about 30% resistance was found. All strains were resistant to beta-lactam antibiotics (benzylpenicillin, ampicillin, carbenicillin and cefuroxime) except cefotaxime. Seven strains (1.6%) were resistant to nalidixic acid. Sensitivity to nalidixic acid is considered an important criterium of CJC.

In 27 patients (13%) antibiotic treatment was instituted; in 12 patients (44%) because they were in a

Table IV. Susceptibility of 435 campylobacter strains to antimicrobial agents (Swedish break points)

	Sen- sitive	Inter- mediate	Re- sistant
Nalidixic acid	98.4	—	1.6
Clindamycin	69.9	28.3	2.8
Erythromycin	97.4	0	2.6
Doxycycline	97.7	0.3	2.0
Gentamicin	100	—	0
Chloramphenicol	98.0	—	2.0
Metronidazole	68.5	—	31.5
Cefotaxime	21.0	40.7	38.3
Other β -lactam antibiotics ^a	0	0	100

^a Benzylpenicillin, ampicillin, carbenicillin and cefuroxime.

life-threatening state and in 15 (56%) because they excreted the organism for a long time. Chemotherapy was given mostly as erythromycin (56%) or as doxycycline (26%). Four out of 22 patients given either of these drugs excreted CJC during the first days of treatment. After 7 to 10 days of therapy CJC could not be recovered in stools from any of these patients. Stool cultures collected from 20/22 patients within 2 weeks after treatment remained negative.

DISCUSSION

CJC is now recognized as a major bacterial intestinal pathogen all over the world. It has been stated that CJC is more prevalent in hot climates (4). Nevertheless, in this material gathered from the Malmö urban area on a latitude of 55°, CJC was the most common bacterial cause of acute enteritis with 6.9% of patients with diarrhoea. The other 3 bacterial intestinal pathogens investigated in this study: *Salmonella*, *Shigella*, and *Y. enterocolitica*, had a total incidence of 7.9%.

The isolation rate for CJC in a group of healthy controls was 0.25%. A possible explanation to this figure can be that a high dose of bacteria is required to cause infection.

Salmonella and *Shigella* are mainly imported from abroad with tourists but occasionally outbreaks occur in Sweden (2). *Y. enterocolitica* is predominantly a domestic pathogen with animal reservoirs (24). The present study showed that CJC was as much a domestic pathogen with 53% of patients

infected in Sweden as it was an imported one. During the time of the study no domestic epidemics occurred. The only epidemics recorded were those mentioned during November 1980 with a total of 16 patients. Imported cases outnumbered domestic ones only for patients between 21 and 30 years. However, these ages most probably have the highest travelling frequency.

CJC shows many similarities with *Y. enterocolitica* in being an endemic intestinal pathogen with alimentary spread to humans from a not yet stated reservoir in the nature (12, 17). This implies that all patients, irrespective of previous journey abroad, with acute enteritis should be examined by bacteriological culture of stools for CJC as well. Moreover, a mode of domestic spread is known as CJC is commonly found in pets (17) and in several animals prepared for food (7, 11, 14).

The age group 15–35 years constituted half of all patients. Butzler and Skirrow (4) calculated that there was almost a 1 in 5 chance that a young adult going to the doctor with diarrhoea had enough to be sampled will be suffering from campylobacter enteritis. The present investigation shows a similar incidence. Children below 10 years of age contributed 17% of all cases, and children below one year 4%. These findings demonstrate that it is also worth while to examine stool samples from small children with diarrhoea for pathogenic bacteria.

In general, campylobacter enteritis is not a severe disease for young and middle-aged people and only 11% of those attending the Department for Infectious Diseases were admitted to hospital. However, most of the hospitalized patients were elderly and were admitted because of severe illness. One 95-year-old patient died of bronchopneumonia and campylobacter enteritis.

84% of the patients studied complained of colics or other abdominal pains and 12% had fresh blood in their stools. These signs and symptoms were probably the major reason for seeing a doctor. It is not known how many patients did not see a doctor. We believe that only a minor part of the cases are diagnosed.

More than 80% of the patients were permanently negative in stools within a month. If chemotherapy as erythromycin or doxycycline was given and the strain was sensitive no relapses occurred when stools from the patients were analysed within 2 weeks after treatment.

Some patients needed antibiotic treatment either

because of life-threatening illness (44%) or long lasting stool carriage (56%). The antibiograms showed that the aminoglycosides, erythromycin, doxycycline, chloramphenicol and nalidixic acid were the most effective drugs. This laboratory has previously reported resistance to erythromycin in 8% of the isolates in 100 consecutive strains with MIC analysis (22). In the present study with 435 consecutive strains, the rate of resistance was only 2.6%, which is almost the same as found for doxycycline and chloramphenicol. There was a high sensitivity to nalidixic acid (98.4%) implying that this drug could be tried for the eradication of organisms in the stool, since plasmid resistance is not induced by this drug. None of the 5 beta-lactam antibiotics tested are considered drugs of choice for campylobacter infections (4).

There were 1.6% of campylobacter strains resistant to nalidixic acid in this material of humans with acute enteritis. Butzler and Skirrow (4) have found that this can be a separate biotype associated with wild birds and particularly seagulls.

The present study has shown that campylobacter is the most common cause of bacterial diarrhoea in Malmö. This is in agreement with another study from western Sweden (18). Domestic cases may outnumber imported ones. This implies that extended stool sampling should be done from domestic patients with acute enteritis.

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Address for reprints:

M. Walder, M.D.,
 Department of Clinical Bacteriology,
 Malmö General Hospital,
 S-21401 Malmö,
 Sweden

Five Cases of *Campylobacter* Jejuni/Coli Bacteremia

MATS WALDER,¹ ANDERS LINDBERG,² CLAES SCHALÉN³ and LENA ÖHMAN¹

From the Departments of ¹Clinical Microbiology, University of Lund, Malmö General Hospital, Malmö

²Infectious Diseases and ³Clinical Microbiology, County Central Hospital, Halmstad, and

³Clinical Microbiology, University of Lund, Lund, Sweden

ABSTRACT. During a 3-year period (1977–1979) 5 cases of *Campylobacter jejuni/coli* (CJC) bacteremia were observed in the south of Sweden. Four of the 5 patients had diarrhoea and 3 of 4 patients tested showed a serological response to a homologous strain. This accumulation of bacteremia cases may be explained from the short duration between the onset of high fever (>38.5°C) and obtaining blood cultures, and the use of blind subcultures in the laboratory procedures.

INTRODUCTION

Recently a number of reports have confirmed that *Campylobacter jejuni/coli* (CJC)¹ (31) is a common cause of acute enteritis in man (3, 6, 10, 26, 30). In contrast to the numerous reports concerning CJC in human fecal flora, there are only a few reports of CJC being found in blood cultures. However, most cases of campylobacter septicemia are caused by other species of campylobacter, e.g. *C. fetus* ss. fetus, which exclusively or preferably attacks compromised hosts. In a review of campylobacteriosis in man, Guerrant et al. (14) described 10 cases of bacteremia caused by CJC. At the same time, 81 cases were found to be caused by *C. fetus* ss. fetus or by non-typable campylobacter.

This report describes 5 cases of campylobacteriosis having positive blood cultures for CJC. These cases are, so far as we know, the first ones in Sweden. Four cases were treated at the County Central Hospital of Halmstad, and appeared from June 1977 to June 1979. The fifth strain was isolated at the Department of Clinical Microbiology, University of Lund, in March 1980.

MATERIALS AND METHODS

Blood samples for culture were collected and handled according to routine laboratory procedure. Following conventional disinfection of the venipuncture site, approximately 3–5 ml of blood was drawn into a sterile syringe and immediately transferred aseptically into a 100 ml culture bottle with 25 ml of nutrient broth, and to an agar slant of 25 ml. The composition of the nutrient broth

was beef extract "Lab-Lemco" powder (Oxoid) 10 g, peptone Bact. Neutr. (Oxoid) 10 g, sodium chloride 5 g, and distilled water 100 ml. The broth was sterilized by autoclaving at 121°C for 40 min at pH 7.1. 10 ml Iso-Vitalex solution and 1 ml Penase (Leo) solution 30 IE/ml was added aseptically. The composition of the agar slant was Brain heart infusion agar (Difco) 52 g, potassium nitrate 0.5 g, hemin solution 0.2% 20 ml, p-aminobenzoic acid 0.2 g, glucose 0.5 g, Liquoid (Roche) 0.5 g and distilled water 1000 ml. The agar was sterilized by autoclaving as above (E. Falsen, personal communication).

Subculture was performed when obvious growth was observed and routine ("blind") subcultures of grossly negative cultures were performed after 4 days at 37°C for 48 h on both horse blood agar and hematin agar plates incubated anaerobically and in 8% CO₂, respectively.

Identification of strains classified as CJC was carried out according to Véron and Chatelain (31). Serum agglutination titers were determined by mixing dilutions of patient serum with an overnight formalin 0.4% treated CJC strain isolated from the same patient.

RESULTS

Identification

All isolated strains of CJC were found to be gram-negative spirally curved motile rods with optimal growth in a 5% oxygen atmosphere. They were catalase and oxidase positive, grew well at 37°C and 42°C and did not ferment or oxidize sugars. They were all susceptible to nalidixic acid as determined by the disc diffusion technique.

¹ The nomenclature used in this communication conforms to the Approved list of bacterial names (27).

CASE REPORT

Case 1

A 56-year-old woman was admitted to hospital with complaints of chest pain, followed some hours later by onset of high fever, headache and diffuse abdominal pain. On arrival she was pale, lethargic and suffering from a slight tenderness over the left lumbar triangle. Blood pressure was 140/75 mmHg and rectal temperature 40°C. Electrocardiogram, chest X-ray and cerebrospinal fluid analyses were normal. Admission laboratory tests showed ESR 43 mm/h and WBC 13 500 with 90% neutrophils; results of other tests were within normal limits.

The initial diagnosis was septicemia. After blood and urine samples for culture had been collected, treatment was begun with ampicillin 2 g intravenously every 6 h for 5 days and orally for another 5 days.

During the first 3 days the patient had several loose stools and moderate abdominal pain but improved continuously. On the 4th day she was afebrile and without complaints. She was discharged on the 10th day after admission.

At a follow-up visit 2 weeks later the patient was healthy and had a normal ESR.

Two out of 3 of the blood cultures taken on admission yielded growth of CJC, which were resistant to beta-lactam antibiotics. Urine culture was negative. Serology for salmonella and yersinia was negative. Agglutination against the patient's own CJC strain was positive in a dilution of 1:128 3 weeks after admission, rising to a titer of 512 6 weeks later.

The patient had not been abroad for the last 3 weeks and repudiated any contact with animals.

Case 2

An 11-year-old girl, previously healthy, fell ill after a one-week stay on the island of Madeira. Following 3 days of diarrhoea, she developed fever and chills, and was finally sent to hospital. The patient complained of abdominal discomfort and had a temperature of 40.0°C. Otherwise physical examination was normal. On admission her laboratory tests showed ESR 42 mm/h and WBC 10 900 with 74% neutrophils.

A preliminary diagnosis of salmonellosis was made and cultures were taken from blood and faeces. The girl was afebrile on the following day. Her diarrhoea vanished after merely dietary treatment. She was discharged on the 4th day after admission. At a follow-up control 2 weeks later, the patient was healthy and ESR was normal.

Stool cultures from the first 3 days were negative for salmonella, shigella and yersinia. CJC in the stool was not analysed. Three blood cultures taken at admission yielded growth of CJC. The patient serum was tested for ability to agglutinate her own campylobacter strain. In a serum drawn 7 days after the patient became ill the titer was 32 and 11 weeks later it was <2.

Case 3

A 74-year-old man had been operated on 4 years previously for a thymoma. During the following years, he was extensively investigated and treated for hypogammaglobulinemia, bone marrow depression with hypoplastic

anemia and neutropenia. He also suffered from a malabsorption syndrome, chronic bronchitis and several episodes of pneumonia.

This "compromised host" was admitted to hospital with a diagnosis of new pulmonary infiltrates. Treatment with oral penicillin was initiated. After 2 days he suddenly developed nausea, vomiting, shivering and spiking fever reaching 39.7°C. He had no diarrhoea. Treatment with intravenous ampicillin and intramuscular gentamicin were initiated after collection of samples for blood cultures.

The patient steadily improved and was afebrile on the 4th day after admission, continuing with oral ampicillin for another 6 days. He remained in fairly good health during the following 8 months and was regularly treated with gammaglobulin.

Bacterial culture of sputum yielded *Haemophilus influenzae* and 2 of 6 blood cultures revealed CJC that were resistant to beta-lactam antibiotics but sensitive to aminoglycosides. In one of the anaerobic blood cultures *Bacteroides necrophorus* was found.

No agglutination titer against the patient's own strain of CJC could be detected.

Case 4

A 66-year-old male smoker suffering from chronic bronchitis and intermittent claudication was admitted to hospital on account of high fever (39.0°C), dyspnea and a severe cough. Physical examination was compatible with a diagnosis of pneumonia but the acute X-ray showed only emphysema. His blood pressure was 140/80, pulse 100, and an abdominal examination was normal. Laboratory studies revealed WBC 14 200 with increased neutrophils and ESR 21 mm/h. Other tests remained within normal limits.

Cultures were taken from blood, pharynx and urine and treatment started with i.v. penicillin for suspected exacerbation of his bronchitis. The patient's temperature gradually fell to normal but on the 3rd day after admission he developed frequent watery diarrhoea. However, he rapidly improved and in spite of a remaining mild diarrhoea he was discharged on the 6th day after admission.

In one of the 3 blood cultures taken on the day of admission, CJC was isolated and in one of 2 stool cultures taken 9 days after the patient had left hospital, CJC was isolated as well. Both isolates were resistant to beta-lactam antibiotics. All other cultures were negative. Serum agglutination against the patient's own strain showed a rising titer from 8 2 weeks after admission to 64 3 months later.

Case 5

A 51-year-old man with a known peptic ulcer disease and repeated bronchopneumonia fell ill with a moderate fever of 38°C and watery diarrhoea. During a one week period without seeing a doctor, the patient's condition worsened. He developed a high fever (40.5°C), chills, headache and muscular pains, but the frequency of diarrhoea decreased. After 2 days of high fever the patient fainted and he was taken to hospital.

On arrival physical examination revealed general distress. He had chills and a temperature of 39.5°C, blood pressure 120/80, pulse 120, and signs of bilateral basal

Table I. Summary of case histories of 5 patients with CJC bacteremia

Ampi = ampicillin, PcV = phenoxymethylpenicillin, genta = gentamicin, PcG = Benzylpenicillin

Case no.	Age	Sex	Other diseases	Temperature		Diarrhoea		Symptoms	Treatment	Antibody titer
				Max. (°C)	Duration of >38.5°C before blood-culture (days)	pres-ent	Duration before blood-culture (days)			
1	56	F	No	40.0	0.5	Yes	Onset later same day	Abdominal pain, headache, nausea, muscular pain	Ampi i.v. 2 g×4	128-512
2	11	F	No	40.0	Hours	Yes	3	Shiverings, abdominal pain	No	32
3	74	M	Thymoma, hypoplastic anemia, malabsorption	39.7	Hours	No	—	Shiverings, vomiting, nausea, respiratory distress	PcV, ampi i.v., genta i.m.	Neg
4	66	M	Chronic bronchitis Heavy smoker	39.9	1	Yes	Onset 3 days later	Respiratory distress cough	PcG i.v.	8-64
5	51	M	Ulcer, broncho-pneumonia	40.5	2	Yes	7	Abdominal pain, headache, muscular pain	Ampi i.v.	Not done

bronchopneumonia. The throat was injected. An abdominal examination revealed normal findings.

A chest X-ray taken on admission was negative and the laboratory examination showed ESR 24 mm/h and WBC 14600 with 88% neutrophils. Other tests, including cerebrospinal fluid analysis, were normal.

The patient was treated with ampicillin i.v. for 5 days and orally for 2 days. He was afebrile after 2 days of treatment and could be discharged on the 7th day after admission.

Cultures from urine and throat were negative, but CJC was isolated from one of 2 blood cultures that were taken on admission. The isolated strain was resistant to beta-lactam antibiotics. Later stool samples showed no growth of CJC. CJC antibody tests were not performed.

The 5 case histories are summarized in Table I.

DISCUSSION

Campylobacter fetus (earlier designated *Vibrio fetus*) infection in man was first described by Vincent et al. in 1947 (34), who isolated campylobacter in cultures taken from the blood of 3 pregnant women of whom 2 aborted later. *C. fetus* ss. *fetus* is the most common etiological agent of systemic campylobacteriosis and the cause of a variety of infections (4, 16). The most common form is a pure septicemia without dissemination, fever being a constant and frequently sole feature (21). The organism can often be isolated from blood cultures.

Five other clinical groups were distinguished by Bejot (1). There are carditis, meningitis, arthritis, other pyogenic processes and abortion, all of them associated with bacteremia. *C. fetus* ss. *fetus* is considered to be an opportunist, chiefly attacking debilitated patients with impaired defences against infection (24). A variety of predisposing diseases are described in the literature e.g. cirrhosis, colon carcinoma (8), Hodgkin's disease (18), agammaglobulinemia (35), chronic lymphatic leukemia (9), Waldenström's macroglobulinemia (22) and lymphoma (23).

In 1957, King (17) described a "related" form of campylobacter causing enteritis in infants and young children. In the few cases of enteritis caused by "related" campylobacter published before 1973 (11, 12, 17, 19, 25, 32) the organism was isolated from blood cultures. Investigations by Butzler et al. in 1973 (5) and Skirrow in 1977 (28) have established a connection between acute enteritis and CJC infection. In certain areas CJC is today the most common bacterial intestinal pathogen causing enteritis (30).

However, in a literature review in 1978 Guerrant et al. (14) found 10 of 91 reported campylobacter bacteremia cases being caused by CJC. The year before, Butzler et al. (7) reported in a review from

Belgium 2 cases of CJC bacteremia during a 5-year period. Between March 1977 and December 1980 the Communicable Disease Surveillance Centre in United Kingdom (Public Health Laboratory Service) received reports of 37 cases of CJC bacteremia among 25 791 campylobacter infections from the whole Great Britain (10). CJC was isolated in both blood and faeces from 21 patients. Another 3 cases of bacteremia and diarrhoea with CJC have been described (13, 15, 26). A few patients with CJC bacteremia, but without symptoms, have also been reported. White (33) drew attention to a 70-year-old man with purulent arthritis and Darrell et al. (11) mentioned a 78-year-old man with cirrhosis and periods of recurrent abdominal tenderness. Two cases of meningitis due to CJC have also been described (20, 29).

We report 4 cases of bacteremia with CJC during a 2-year period from one Swedish hospital. A 5th case of bacteremia with CJC from another Swedish hospital is also presented. Four of the 5 cases suffered from diarrhoea. The clinical signs were characteristic of CJC infection with high fever, abdominal pain and frequent watery diarrhoea. One of the 5 patients, a compromised host, had no gastrointestinal symptoms, but on the other hand, showed signs of septicemia with high spiking fever, discomfort and chills. This patient did not show a serological response which was probably due to his underlying disease, hypogammaglobulinemia. Of the other 3 patients tested, all showed an antibody response to their homologous strain.

Since CJC is a pathogen or a commensal in many animals (cattle, sheep, swine, poultry, dogs and cats), food, milk, water, and sick animals are major sources of infection (3, 6, 7). None of our patients had contact with farms, animals or agricultures; one returned from a holiday to Madeira. Other sources of infection could not be established. A previous study revealed an even distribution of infections between rural and urban dwellers (30).

Bacteremia with CJC is probably more frequent than clinically suspected since systemic invasion is one of its pathogenic mechanisms in the early stage of the disease; other reports are in agreement with this view (2, 6). Hitherto no ST (heat-stable) or LT (heat-labile) toxin has been reported, but as campylobacter has LPS (lipopolysaccharide) endotoxic activity may exist, accounting for some of the septic symptoms.

One reason for the scarce isolation frequency of

CJC in blood is the weak growth in liquid medium. Moreover, the lack of blood culturing from patients with common enteritis explains the predominance of species other than CJC found in campylobacter bacteremia. Reasons for the accumulation of bacteremia cases reported here should be sought for in the clinical routine of blood culturing from patients with unknown high fever or in the laboratory procedures, including the media used. CJC is a fastidious organism only growing as a thin veil in liquid media and thus, agar slant and blind subcultures are needed to detect its growth. The automatic Bactec 460 system is likely to prove a great help in the detection of CJC in blood cultures (26).

We postulate a more frequent isolation rate of CJC from the blood of enteritis patients if blood samples for culture are taken in the early stages of the disease and if cultures are properly analysed.

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Address for reprints:

M. Walder, M.D.,
Klin. Bakt. Lab.,
Allmänna Sjukhuset,
S-21401 Malmö

DIARRHOEA IN SWEDISH INFANTS

Aetiology and Clinical Appearance

B. L. PERSSON, A. THORÉN, B. TUFVESSON and M. WALDER

From the Departments of Paediatrics and Infectious Diseases, Sections of Clinical Virology and Bacteriology, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden

ABSTRACT. Persson, B. L., Thorén, A., Tufvesson, B. and Walder, M. (Departments of Paediatrics, Infectious Diseases, Sections of Clinical Virology and Bacteriology, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden). Diarrhoea in Swedish infants. *Acta Paediatr Scand*, 71, 1982.—Ninety-five children with gastroenteritis admitted at the Department of Paediatrics, Malmö General Hospital, Malmö, Sweden, starting November 1978 through October 1979 were studied. Patients were examined clinically and the course of their disease was followed. Stool specimens from all patients were analysed by routine bacteriology and rotavirus identification was done by immune-electroosmophoresis. Fifty-eight per cent of patients had rotavirus. *Campylobacter* was found in 6 %, enteropathogenic *E. coli* in 2 %, *Yersinia enterocolitica* in 2 % and *Salmonella* in 1 % of patients. Stool specimens from 53 healthy children matched for sex and age served as controls for bacterial pathogens. All were negative. Forty per cent of patients presented with a clinical dehydration, but only 11 %—half of them with bacterial enteropathogens—needed i.v. therapy and 4 % required antibiotic treatment. Median time for hospitalization was three days. Vomiting, fever, watery diarrhoea and initial clinical dehydration were more commonly associated with rota virus infection. Macroscopic blood in stools was restricted to patients with bacterial aetiology.

KEY WORDS: Infants, infectious diarrhoea, rota virus, *Campylobacter jejuni*, enteropathogenic *E. coli*, *Yersinia enterocolitica*

In recent years our knowledge of the aetiology of diarrhoeal diseases in children has expanded with the discovery of new viral and bacterial microorganisms such as rotavirus, enterotoxigenic *E. coli* (ETEC) and *Campylobacter jejuni*. The management of diarrhoeal disease has also focused interest since oral rehydration therapy seems to be effective even in severe cases such as cholera. Accordingly, WHO has called for epidemiological studies of infantile diarrhoea from different parts of the world (1). Recently such studies have been published from Finland (2, 3). The aim of this study was to establish the role of "newer" enteropathogens (rotavirus and *Campylobacter jejuni*) in relation to previously known agents such as for instance enteropathogenic *E. coli* (EPEC) and *Yersinia enterocolitica*.

Further we wished to correlate the clinical signs and symptoms with aetiology.

METHODS

Patients

The study was performed from November 1978 through October 1979 at the Paediatric Clinic, Malmö General Hospital, Malmö, Sweden. During this period, diarrhoea was the main diagnosis in eight per cent of all inpatients. Ninety-five consecutive cases admitted for diarrhoea were included in the study. Patients were admitted to hospital when oral rehydration at home had failed or seemed hazardous due to vomiting and/or dehydration. Fifty-three healthy children, matched for age and sex, attending of the clinic's health service during the same period served as controls for carrier-state of bacterial pathogens. Rotavirus were not investigated among controls. Clinical data were obtained including age, sex, duration of symptoms before hospital contact, appearance of stools (graded as watery or not watery) and the presence of fever, vomiting and concomitant respiratory symp-

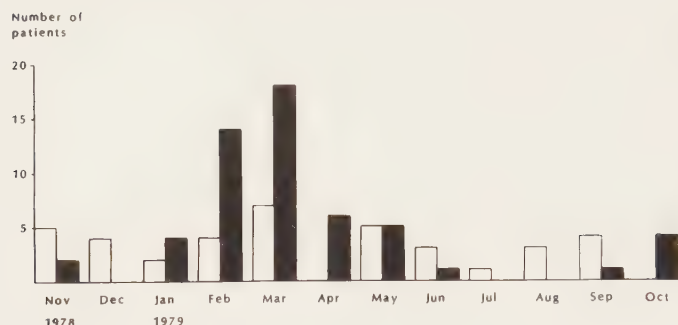


Fig. 1. Monthly distribution of patients with diarrhoea. □, rotavirus-negative patients; ■, rotavirus-positive patients.

toms. Dehydration was graded as mild, moderate or severe (4). The duration of hospital stay as well as intravenous treatment and antibiotic therapy given for the diarrhoeal disease were recorded.

Oral rehydration therapy was carried out as follows: during the first 12 to 24 hours fluid was given as slightly sweetened tea or carrot soup plus table salt (Na^+ about 60 mmol/l). The volume corresponded to 125 ml/kg bodyweight per 24 hours divided in 5–6 meals. This initial nutrition pause was followed by successive introduction of nutrients in a glutenfree diet during a five day period.

The following children were excluded from the study: patients with nosocomial infections, patients on broad-spectrum antibiotics or erythromycin and patients with food intolerance.

The chi-square test was used in the statistical analyses.

Laboratory methods

Stool samples taken as rectal swabs were obtained from all patients and controls. They were cultured by current methods for *Salmonella*, *Shigella*, *Campylobacter jejuni*, *Yersinia enterocolitica* and EPEC (5, 6).

The EPEC antisera were manufactured by the National Bacteriological Laboratory (NBL), Stockholm, Sweden and contained the following O-serogroups: O26, O44, O55, O78, O86, O111, O114, O119, O124, O125, O126, O127, O128. The serogroup O78 has been connected with epidemic infantile diarrhoea (7) but is today more frequently reported as enterotoxin producing strain, ETEC. The EPEC isolates were tested for production of heat labile enterotoxin (LT) performed at NBL, production of heat stable enterotoxin (ST) and invasive capacity (8, 9, 10). For analysis of rotavirus, a 1 ml volume of stools were analysed (11).

RESULTS

Aetiology and source of infections

All cases of diarrhoea were of domestic origin with the exception of three patients returning from the Mediterranean area. Two of them had *Salmonella* and *Campylobacter* respectively while no diagnosis could be established for the third patient.

Isolation rates of intestinal pathogens are shown in Table 1. Rotavirus being the the most important cause of diarrhoea in this study was found in 58% of the cases, all infected in Sweden.

Campylobacter was found in six patients. One of them had a triple infection of *Campylobacter*, rotavirus and EPEC, another *Campylobacter* + rotavirus. Two patients had EPEC infection, the one previously mentioned of serogroup O127: B8, the other one O78. The EPEC strains were negative concerning enterotoxin production (LT + ST) and invasive capacity. Another two patients had *Yersinia enterocolitica* serotype 3. There was one patient with *Salmonella*, but no one with *Shigella*. No bacterial enteropathogens were found among the controls.

Seasonal and age distribution

Monthly distribution of patients with diarrhoea is shown in Fig. 1, the peak rate occurring in the months of February and March.

Table 1. Aetiology of patients with diarrhoea

	Patients (n=95)
Rotavirus	55 (58%) ^{a+b}
<i>Campylobacter</i>	6 (6%) ^{a+b}
EPEC	2 (2%) ^b
<i>Yersinia</i>	2 (2%)
<i>Salmonella</i>	1 (1%)
Unknown aetiology	32 (34%)

^a One patient with Rotavirus and *Campylobacter*.

^b One patient with Rotavirus, *Campylobacter* and EPEC.

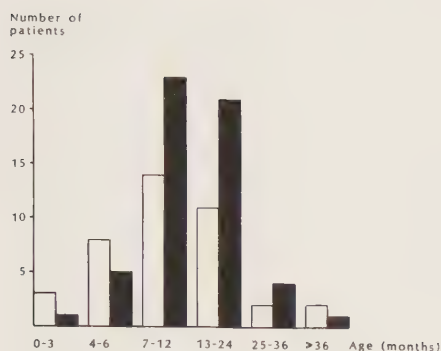


Fig. 2. Age distribution of patients with diarrhoea. □, rotavirus-negative patients; ■, rotavirus-positive patients.

Distribution of sex was equal among patients. According to Fig. 2, 91% were under two years of age (median age 11 months, range 1 month–4 years).

Clinical findings

Fig. 3 shows the presenting clinical symptoms. Acute symptoms such as fever, vomiting, watery diarrhoea and dehydration were more common with rotavirus infections. Associated respiratory symptoms were, however, not more common with rotavirus patients. Median duration of symptoms before hospital contact was 2 days (range 1–20 days). Seven patients had protracted diarrhoea more than 14 days. Duration of symptoms before and after hospital contact was not correlated to aetiology.

Macroscopic blood in stools was only observed in patients with bacterial aetiology—one with *Salmonella*, two with *Campylobacter*. A mild dehydration was found in four of the children with *Campylobacter* infection and the patient with *Salmonella*. Otherwise, the presenting clinical symptoms of patients with bacterial pathogens did not differ from other patients.

Treatment

Treatment was generally symptomatic, and specific antimicrobial therapy was only given

to four patients—the two with *Yersinia* infection, one with *Campylobacter* and one with unknown aetiology. Antibiotics were given because of proved (or in one case suspected) bacterial aetiology when the clinical response was poor after 2–3 days of initial intravenous rehydration therapy. Oral rehydration was generally successful and only in ten (11%) of the patients was i.v. therapy needed; rotavirus—four, *Campylobacter* + rotavirus—one, *Campylobacter*—two, *Yersinia*—two, unknown aetiology—one. The median duration of hospital care was three days (range 1–15 days).

DISCUSSION

Children presenting with symptoms of gastroenteritis such as fever, vomiting and diarrhoea give very few discriminating signs regarding the aetiology of the disease. The difference between rotavirus patients and others in our study was, in this aspect, not impressive. Abdominal pain is difficult to evaluate in small children. Other clinical signs are probably more related to the age of the patients than to the aetiological agent. Macroscopic blood in stools was a rare finding, only seen in

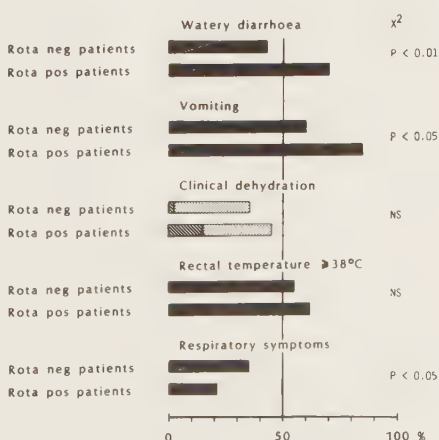


Fig. 3. Clinical signs and symptoms of patients at hospital contact (%). Clinical dehydration: ■, moderate; □, mild.

three patients, two of them with *Campylobacter* infection. For children under two years of age presenting with bloody diarrhoea and abdominal pain the differential diagnosis of *Campylobacter* infection versus intussusception must be kept in mind (12).

In epidemiological investigations of diarrhoea in children some years ago there was generally a low isolation rate of intestinal pathogens (13). After the discovery of rotavirus, it was shown that this agent was the most common cause of childhood diarrhoea both in industrial and developing countries (2, 14, 15).

The monthly distribution of diarrhoea found in this study corresponds with earlier observed winter-peaks of rotavirus infection (16, 17). Previous perennial studies indicate that rotavirus causes 28–65% of the hospitalized cases of childhood diarrhoea in temperate areas (17, 18, 19), compared to the 58% found in Malmö.

Clinical findings such as fever, vomiting, watery diarrhoea and dehydration are commonly seen, although the disease is generally mild in temperate areas (16, 17, 19, 20). Respiratory symptoms, however, did not correlate to rotavirus in this study. In contrast Lewis et al. reported respiratory symptoms in two third of rotavirus cases (21).

In contrast to rotavirus (58%), bacterial enteropathogens were comparatively rare (11%) among inpatients. In comparison, from November 1978 through July 1979 bacterial pathogens were detected in only 6% of 82 outpatients (unpublished data). It is of interest that two out of three inpatients infected abroad had bacterial pathogens. In addition the two non-domestic cases of the 82 outpatients both had bacterial enteropathogens—one EPEC O26: B6 the other *Campylobacter* + EPEC O126: B16 (unpublished data).

Campylobacter as a cause of diarrhoea is found in all age groups but according to Butzler especially in infants under two years of age (22). In developing countries high relative frequencies of *Campylobacter* infections

have been reported for infantile diarrhoea, 13–35% (15, 23). In industrialized countries corresponding frequencies of 4–6% have been reported from Belgium and Canada (24, 25). In a recent Swedish study including 435 patients with *Campylobacter* infection, 11% were under two years of age. The relative frequency of *Campylobacter* enteritis was 7% (5). *Campylobacter* is thus the most common bacterial agent associated with infantile diarrhoea in Sweden. In general, EPEC infections nowadays seem to be mild and rare in industrialized countries in contrast to countries with poor sanitary conditions (26). However, in a recent study from Finland, EPEC was still the most frequent bacterial pathogen encountered (3). The pathogenesis of EPEC diarrhoea remains obscure, and our isolates were negative concerning enterotoxin production and invasive capacity. Patients were not investigated for presence of enterotoxin producing strains belonging to other serogroups (ETEC). However, ETEC strains seems to be rare in Scandinavia (3, 27).

In these Swedish children, having a good state of nutrition, the clinical symptoms of diarrhoea were comparatively mild. Rehydration therapy could generally be administered orally and few patients received antibiotics. Due to the rather nonspecific clinical signs of infantile gastroenteritis, stool sampling is essential, in order to establish the local epidemiological pattern and to identify the aetiology of those critically ill patients who would benefit from specific antibiotic therapy.

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(M. W.) Section of Clinical Bacteriology
Department of Medical Microbiology
Malmö General Hospital
21401 Malmö
Sweden

Aetiology and Clinical Features of Severe Infantile Diarrhoea in Addis Ababa, Ethiopia

by ANDERS THORÉN^{1,2,4}
GUDMUND STINTZING^{1,5,6}
BERTIL TUFVESSON³
MATS WALDER² and
DEMISSIE HABTE¹

Infantile diarrhoea remains one of the leading causes of childhood morbidity and mortality, being most prominent in developing countries. WHO has accordingly underlined the need for epidemiological surveys of infantile diarrhoea in different geographical areas.^{1,2} Children under the age of 2 years are the most commonly affected. The spectrum of recognized intestinal pathogens has increased following recognition of agents such as rotavirus, enterotoxigenic *Escherichia coli* (ETEC) and *Campylobacter fetus* ss *jejuni*. Several epidemiological investigations have been carried out in developing countries during the last few years in order to establish the role of rotavirus and ETEC.³⁻⁶ Regarding *Campylobacter* infections, however, there are currently only a few reports concerning its role in developing countries.^{7,8} In the City of Addis Ababa infantile diarrhoea is a very serious health problem. In a previous investigation, on patients attending the Outpatients department, we demonstrated the importance and seasonal occurrence of rotavirus and enterotoxigenic bacterial infections in this area.⁴

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¹The Ethio-Swedish Pediatric Clinic and Department of Child Health, Faculty of Medicine, Addis Ababa, Ethiopia (The Departments of Bacteriology², Virology³ and Infectious Diseases⁴, General Hospital, S-214 01 Malmö, Sweden).

⁵The National Bacteriological Laboratory, Stockholm, Sweden.

⁶Department of Pediatrics, St. Görans Hospital, Stockholm, Sweden.

Correspondence to Dr. Anders Thorén, Department of Infectious Diseases, General Hospital, S-214 01 Malmö, Sweden.

The aim of this investigation was to identify the aetiology of diarrhoea in children admitted for intravenous rehydration therapy, and make an attempt to correlate clinical features with aetiology.

Material and Methods

Patients and Controls

Children with diarrhoea ($n = 175$) admitted to the rehydration unit of the Ethio-Swedish Pediatric Clinic, Addis Ababa, Ethiopia (March through June 1979) were included in the study. The study was performed in the beginning of the rainy season which is also the peak season for diarrhoeal diseases.⁴ Children without diarrhoea ($n = 74$) attending the clinic served as controls. The controls were matched for age and sex. Patients treated with broadspectrum antibiotics within one week before admission were excluded. A history, including feeding habits and duration of the presenting symptoms was obtained from the mother by a specially trained nurse. Physical examination was done by one of us (G.S.). This included assessment of nutritional status,⁹ degree of dehydration and signs of concomitant diseases. Rectal temperature and quality of stools were recorded. The quality was graded as watery or not watery, the latter group including loose, mucoid and/or bloody stools. The time required for parenteral hydration therapy was also noted.

Handling of Stool Specimens

Stool specimens were obtained directly on admission and microscopic examination for parasites plus a leucocyte and erythrocyte count was performed on fresh preparations. Only trophozoites of *Giardia lamblia* and *Entamoeba histolytica* were accepted as relevant findings for diarrhoea. Stool samples were also sent on modified Stuart medium¹⁰ to the National Bacteriological Laboratory (NBL) Stockholm, Sweden for enterotoxin bioassay (LT) and to the bacteriological laboratory, General Hospital, Malmö, Sweden for identification of

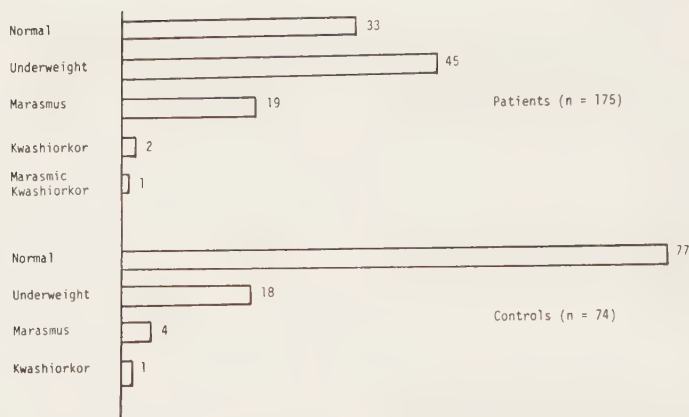


Fig. 1. Nutritional status of patients and controls (%).

Salmonella, *Shigella*, enteropathogenic *E. coli* (EPEC), *Campylobacter fetus ss jejuni*, *Yersinia enterocolitica* and *Vibrios*. All specimens were cultured within 10 days from collection. Stool specimens were also sent to the department of Virology, General Hospital, Malmö, Sweden for rotavirus analysis.

Laboratory Methods

Shigella was cultured on Xylose-Lysine-Desoxycholate agar (XLD-BBL). Enrichment cultures for *Salmonella* were performed in Rappaport tubes for 18 h followed by culture on Lactose-Sacharose-Urea agar, LSU-agar.¹¹ *Yersinia enterocolitica* was cultured on LSU-agar incubated for 18 h in 25°C and also in enrichment broth: 18 h in 5 ml tubes containing one antibiotic disc with carbenicillin (100 µg) and one with cephalothin (30 µg). The broth was then plated on LSU-agar incubated 18 h at 37°C. *Vibrios* were cultured using enrichment procedure for 18 h in alkaline peptone water followed by culture on blood agar and thiosulphate-citrate-bile-salt-sucrose (TCBS) agar. Identification of heat-labile enterotoxin (LT) producing strains was done using the Y1 adrenal cell test as described previously.¹² Only strains giving a positive reaction with >50% of the adrenal cells showing a typical rounded appearance were considered positive.

For isolation of EPEC, 5 lactose positive colonies of *E. coli* were picked from each specimen and subcultured on blood agar after which agglutination was done with polyvalent and monovalent antisera (Wellcome Reagent Ltd, Colindale, England). The antisera contained the following serogroups: 026:B6, 044:K74 (L), 055:B5, 086:B7, 0111:B4, 0114:K90, 0119:B14, 0124:B17, 0125:B15, 0126:B16, 0127:B8, 0128:B12. Strains were confirmed as EPEC by tube titration after heating to 100°C for one hour.¹³ The

EPEC strains were also tested for heat-stable enterotoxin (ST)¹⁴ and invasive properties.¹⁵ Isolation and identification of *Campylobacter fetus ss jejuni* was done according to Skirrow 1977¹⁶. Biochemical identification of all bacterial isolates was performed according to standard methods.¹⁷ Rotavirus was identified by Immune-Electro-Osmo-Phoresis, IEOP.¹⁸

Results

Patients and Controls

Ninetyeight per cent of the patients were under 2 years of age, 0–6 months—37%, 6.5–12 months—51% and 12.5–24 months—10%.

A high number of patients (47%) and controls (43%) were not breastfed. The difference of patients and controls that received only breastmilk up to 6 months of age was significant: 8% of the patients versus 36% of the controls ($p < 0.01$, χ^2 -test). Children with diarrhoea were significantly more malnourished ($p < 0.001$, χ^2 -test), (Fig. 1).

Aetiology

Intestinal pathogens were found in 122 of the 175 patients (70%), compared to 17 of the 74 controls (23%). From one third of the patients more than one, maximum 3 intestinal pathogens were isolated at the time of investigation.

Table 1 shows the isolation rates of different intestinal pathogens. Rotavirus was the most frequent finding encountered in nearly half of the patients. Bacterial pathogens were isolated from 43% of the patients with EPEC as the most frequent finding (19%). Altogether 9 different serogroups were found in patients and controls, representing the following serogroups: 044:K74 (L) 4, 055:B5 9, 086:B7 1, 0111:B4 11, 0114:K90 2, 0119:B14 2,

Table 1
Isolation rates of different enteropathogens

	Patients (n = 175)		Controls (n = 74)		
	No	%	No	%	
Enteropathogenic <i>E. coli</i>	33	19	6	8	p < 0.05 (χ^2 -test)
Enterotoxigenic bacteria	15	9	5	7	N.S. (Fischers exact test)
<i>Campylobacter</i>	22	13	2	3	p < 0.01 (Fischers exact test)
<i>Shigella</i> spp	3	2	0	0	—
Non Cholera <i>Vibrio</i>	1	1	0	0	—
Rotavirus	85	49	4	5	p < 0.001 (χ^2 -test)
<i>G. lamblia</i> *	4	2	1	1	—
<i>E. histolytica</i> *	2	1	0	0	—
No enteropathogens discovered	53	30	57	77	

* Only findings of trophozoites recorded in the table.

0126:B16 4, 0127:B8 6, 0128:B12 1. Two EPEC strains of serogroup 0114:K90 produced heat-labile enterotoxin (LT). All EPEC strains were negative when tested for heat-stable enterotoxin (ST) and invasive properties.

Enterotoxin producing strains (LT) were found in 9% of the patients. Altogether 27 strains were isolated from patients and controls, 22 of these were *E. coli* and 5 represented other enterobacteriaceae (*Citrobacter freundii* 3, *Enterobacter agglomerans* and *Klebsiella pneumoniae*). In one patient 3 different ETEC strains were isolated, while in 2 patients ETEC plus enterotoxigenic non-*E. coli* were isolated. One control case had 2 different ETEC strains, and another an enterotoxigenic *Citrobacter freundii* as the only finding.

No samples were found to contain *Salmonella*, *Yersinia enterocolitica* or *Vibrio cholerae*. One patient had a non-cholera *Vibrio*.

Clinical Symptoms Related to Aetiology

Quality of stools, duration of diarrhoea, rectal temperature and severity of dehydration are related to aetiology in Table 2. Watery diarrhoea and fever was common with EPEC patients while not watery diarrhoea was more common with ETEC patients, although these differences were not significant.

Faecal counts of leucocytes and erythrocytes did not correlate to any specific intestinal pathogen with the exception of the few patients with *Shigella* who all had high leucocyte counts. Neither did age, breast-feeding habits, nutritional status or time needed for parenteral hydration therapy show any correlation to aetiology. The clinical features of diarrhoea were similar in patients having one intestinal pathogen only, compared to patients with double infections.

Five patients died (3%), 4 of them were malnourished and not breastfed. All 5 had rotavirus infection, one also had *Campylobacter* and 2 had EPEC.

Discussion

Severe infantile diarrhoea caused by certain bacterial pathogens such as *Shigella*, EPEC and possibly *Campylobacter* is an indication for antimicrobial therapy^{13,16,19}. In this regard it was interesting to investigate the aetiology of diarrhoea in hospitalized children and relate clinical parameters to aetiology. Concerning the latter aspect, results were discouraging, suggesting that the clinical features of infantile diarrhoea are rather nonspecific when related to aetiology.

The microbiological investigations yielded a high

Table 2
Quality of stools, duration of diarrhoea, fever and dehydration related to aetiology.
(Patients with mixed infections were excluded)

	Watery diarrhoea	Diarrhoea > 14 days	Temperature $\geq 38.0^\circ\text{C}$	Severe dehydration
Enteropathogenic <i>E. coli</i>	9/10	1/10	7/10	3/10
Enterotoxigenic bacteria	2/8	1/8	3/8	4/8
<i>Campylobacter</i>	5/10	1/10	4/10	3/10
Rotavirus	28/47	6/47	16/47	8/47
All diarrhoeal cases	105/175	24/175	67/175	30/175

total isolation rate (70%) compared to many previous studies. Still 30% of the diarrhoeas are aetiologically unexplained. Part of this gap might be filled when other recently discovered viral agents are included in comprehensive studies. The relatively high isolation rate of intestinal pathogens in the control group (23%) underlines the need for control groups in epidemiological studies. The results confirm the important role of rotavirus in this age group. Enterotoxigenic bacteria seem less important particularly since they were also a frequent finding among the controls. In this study the stool specimens were only tested for heat-labile enterotoxin (LT). However, enterotoxigenic strains producing heat-stable enterotoxin (ST) only, seem to be rare in this population (Stintzing *et al.*, unpublished data).

E. coli of classical serogroups (EPEC) was the most frequent bacterial pathogen encountered, as it was in a recent report from South Africa.²⁰ This is interesting since the relevance of EPEC serogrouping in endemic cases of diarrhoea has been under debate.²¹ Also *Campylobacter* infections, of which there are still very few reports from developing countries seems to be an important bacterial pathogen.

In other geographical areas such as Bangladesh, *Vibrio cholerae* is a common bacterial agent.²² Epidemics of cholera have not occurred for many years in Ethiopia (1973) and in this study the only finding was a non-cholera *Vibrio* which probably accounts for some sporadic cases of diarrhoea also in Ethiopia.²³

The vast majority of children were less than 2 years of age. The most affected group, 6–12 months, coincides with the weaning period, and the source of infection is presumed to be contaminated food and water.²⁴ In this aspect early weaning and the increasing incidence of bottle feeding is alarming. The poor nutritional status among patients compared to controls cannot be explained by different breast-feeding habits only. The episode of diarrhoea bringing the child to hospital may have been one of many, with successive impairment of the nutritional status of the child perpetuating the vicious cycle of malnutrition and diarrhoea.

In this comprehensive study, rotavirus, EPEC and *Campylobacter* were the most important aetiological agents. However, to establish the aetiology based on only clinical features seem to be impossible in this area.

The application of new and simple diagnostic techniques in addition to relevant epidemiological information is essential in coping with the problem of infantile diarrhoea in developing countries.² The implications of such obtained results include promotion of breastfeeding, development of the most desirable vaccines, and specified indications for antibiotic therapy.

Summary

This study, performed at Ethio-Swedish Pediatric Clinic, Addis Ababa, Ethiopia, from March through June 1979, included 175 children with diarrhoea in need of parenteral rehydration and 74 age and sex matched controls. Stool samples were analyzed using microscopy, routine bacteriology including culture for *Campylobacter fetus ss jejuni*, enterotoxin bioassay (LT) and rotavirus analysis. The clinical appearance of patients and stools was recorded. A microbiological diagnosis was established in 70% of the patients as compared to 23% positive findings in the controls. The most common findings were rotavirus (49%), enteropathogenic *E. coli* (EPEC) (19%) and *Campylobacter* (13%) of the patients. Among bacterial enteropathogens EPEC was the most prevalent. The isolation rate of enterotoxigenic bacteria was 9% in patients, not differing significantly from the controls, 7%. The correlation of clinical symptoms to aetiology was not conclusive suggesting that the clinical picture of infantile diarrhoea in most cases is nonspecific.

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